STUDIES ON AMMONIA AND LABILE AMINO GROUPS IN PERFUSED RAT HEART

BY

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Thesis submitted for the degree of Doctor of Philosophy

University of Edinburgh September 1975

PREFACE AND ACKNOWLEDGEMENTS

This Thesis is a report of work carried out in the University of Edinburgh, between October 1970 and September 1973, and is my own work. I was supported during this period by a Research Scholarship from the Faculty of Medicine, and many of the materials used were provided from a grant from the Science Research Council.

I am greatly indebted to my Supervisor, Dr. J.H. Ottaway, for his help and encouragement during the performance of the experimental work, and to Professors R.B. Fisher and G.S. Boyd, for giving me the opportunity of working in the Biochemistry Department at Edinburgh.

I would also like to thank Professor D. Pette for the invitation to spend some time in his laboratory in Konstanz, West Germany, and for tuition given by him and Dr. J. Nolte in techniques of enzyme localization.

Finally, I would like to thank Dr. A.E. Stuart and the staff of the University Department of Pathology, Edinburgh, for allowing me the use of facilities for cutting, staining and photographing tissue sections, and Dr. R.O.D. Dixon, of the Department of Botany, for providing facilities for the measurement of ^{15}N-ammonia by mass spectrometry.

I would also like to thank Miss Linda Graham for her patient and skilful typing of this Thesis.

CONTENTS

SUMMARY.

Muscles contain a relatively high concentration of ammonia, which they produce as part of their normal metabolism and release into the bloodstream. The present study is an investigation into the production and metabolism of ammonia by the isolated perfused rat heart.

Ammonia is produced by rat heart perfused with media to which none is added; conversely, ammonia added to the perfusing fluid is taken up and metabolized by the heart. In the first case an equilibrium is reached in which the concentration of ammonia in the intracellular fluid is approximately twenty times that in the perfusate. A similar ratio obtains in the rat heart *in vivo*, as measured by other authors. Variation of the ammonia concentration in the perfusing fluid alters the distribution ratio in a way that is inconsistent with widely-held views that ammonia is freely diffusible throughout the tissue, and that its distribution is controlled solely by passive diffusion across the plasma membrane. It is proposed that the anomalously high internal concentration is maintained in part by an ammonia pump.

The existence of an ammonia pump is supported by experiments with ^{15}N-labelled ammonia, which show that relatively more ammonia enters the heart at low external concentrations than would be expected on the basis of observations at high external concentrations. Labelling of tissue ammonia in these experiments is much less than expected: there appear to be two ammonia pools in rat heart, only one of which exchanges rapidly with the perfusate. Possible locations of these pools are suggested.

The intracellular location of some enzymes connected with the metabolism of ammonia and amino acids in rat heart is also investigated, and the relationship of enzyme distribution to the compartmentation of ammonia and related metabolites is discussed.

An attempt is made to relate the formation and disappearance of ammonia to amino acid metabolism in perfused rat heart. Glutamine is the most likely precursor of the ammonia produced by the heart. It was not possible however to demonstrate the fate of ammonia nitrogen taken up by the heart.

The ammonia concentration in the intracellular fluid is maintained within comparatively narrow limits despite a tenfold variation in perfusate ammonia concentration. Three processes may be involved in control of the intracellular ammonia concentration: synthesis or metabolism of ammonia within the tissue; active uptake of ammonia from the extracellular fluid; and intracellular compartmentation of ammonia. Possible functions of ammonia in rat heart are suggested, including enzyme regulation and the mobilization of weak acid substrates.

CHAPTER 1

GENERAL INTRODUCTION

Chapter 1. General Introduction.

Ammonia is present in all muscle tissue. Some representative concentrations are shown in Chapter 3, Table 3.1. The first extensive investigations into its formation in skeletal muscle were performed many years ago (Embden and Zimmermann, 1927; Parnas and Mozolowsky, 1927), and although a number of reports of ammonia production have appeared since (see below), relatively little is known about the formation and fate of ammonia in other types of muscle, nor about the kinetics of its metabolism. Although the concentration of ammonia in muscle tissues is much higher than that in plasma (see Chapter 3, Section 3.1), mechanisms responsible for maintaining this concentration difference have never been satisfactorily elucidated. The possible functions of such a high concentration in the muscle cell have never been adequately discussed; indeed, ammonia has generally been considered a highly toxic substance (see Section 1.3 below).

A series of early studies on frog skeletal muscle described the magnitude and kinetics of ammonia production under various conditions, and the relation of ammonia to adenine nucleotide metabolism (Embden and Wassermeyer, 1928a,b; Embden et al., 1928a,b; Parnas, 1929, 1932; Parnas and Lewinsky, 1935; Parnas and Mozolowsky, 1927; Parnas et al., 1927, 1934). The production of ammonia by rabbit muscle (Embden and Wassermeyer, 1928b; Embden et al., 1928a) and human forearm (Parnas et al., 1927) was also reported. The relation of ammonia production to muscle metabolism and activity was discussed in a review by Parnas (1935).

More recently, ammonia production by a wide range of muscle

tissues has been described, including rat leg muscle (Brown et al., 1957; Gerez and Kirsten, 1965; Chiosa and Busneag, 1971; Lowenstein, 1971, 1972), dog leg muscle (Duchêne-Marullaz et al., 1964a), human forearm (Allen and Conn, 1960; Duchêne-Marullaz et al., 1964a), stomach muscles from various vertebrate species (Silakova and Bekir-Zade, 1967), and locust muscles (Gerez and Kirsten, 1965). Early experiments with heart muscle showed that ammonia was released by beating frog heart (Ostern, 1930) and by a mammalian heart-lung preparation (Cruikshank and McClure, 1936). More recent research on heart has centred mainly on the clinical and diagnostic importance of myocardial ammonia metabolism. Ammonia production has been described in perfused rabbit heart (Feinberg and Alma, 1960), rabbit heart slices (Trush, 1963a), rabbit heart in vivo (Kato, 1968; Watanabe, 1968; Watanabe et al., 1969, dog heart (Duchêne-Marullaz et al., 1966; Kobayashi, 1967; Aviado et al., 1968) and human heart (Keul et al., 1964, 1966).

1.1 Precursors of Ammonia in Muscle.

Early investigators realized that ammonia was formed in vertebrate skeletal muscle by the breakdown of AMP. This may not be the case in other muscle types such as heart (see Chapter 5). Embden made the first preparation of AMP from rabbit skeletal muscle, established its identity, and proposed it as the precursor of ammonia in this tissue (Embden and Zimmermann, 1927). He showed that frog muscle which was "traumatized" by grinding it in a pestle and mortar liberated ammonia from added adenine nucleotides (Embden et al., 1928a). The quantity of ammonia produced by

traumatized muscle was stoicheiometrically equivalent to the extent of conversion of adenine nucleotides to hypoxanthine nucleotides (Embden and Wassermeyer, 1928b; Parnas, 1929). More recently, conversion of adenine nucleotides to hypoxanthine nucleotides was observed in active frog muscle by Lange (1955) and by Wajzer et al., (1956), and in rabbit muscle by Bendall and Davey (1957). Some workers have suggested that ammonia is formed from adenine nucleotides in rabbit heart (Trush, 1963a; Watanabe, 1968; Watanabe et al., 1969; Kato, 1968), although none of these investigators measured hypoxanthine nucleotides. The discovery of AMP deaminase (Schmidt, 1928) provided an enzymic basis for ammonia production in skeletal muscle:

$$AMP + H_2O \longrightarrow IMP + NH_4^+.$$

The enzyme has since been purified (Kalckar, 1947), crystallized (Lee, 1957; Smiley et al., 1967), and its physical and kinetic properties have been extensively studied (Conway and Cooke, 1939b; Setlow and Lowenstein, 1967, 1968; Lee and Wang, 1968; Ronca et al., 1968).

In traumatized or hypoxic muscle ammonia and hypoxanthine nucleotides are produced in equivalent amounts (Embden and Wassermeyer, 1928b; Parnas, 1929). The adenine nucleotide content of adequately oxygenated muscle on the other hand is not significantly diminished by activity, although ammonia production continues (Parnas, 1932, 1935); indeed the amount of ammonia produced in 24 hours by an isolated beating frog heart can rise to five times the original adenine nucleotide content (Ostern, 1930). It was

therefore postulated that the aerobic muscle could synthesize adenine nucleotides from IMP (Parnas and Lewinsky, 1935). Parnas believed that the amino donor in this reaction was not free ammonia, which diffused out of the cell on production and was carried away by the bloodstream, but was probably an organic amino compound such as an amino acid (Parnas, 1935). The identity of the amino compound remained unknown until Newton and Perry (1960) demonstrated the incorporation of isotope from ^{15}N-aspartate into the 6-amino group of the adenine nucleotides of rabbit muscle. They isolated an intermediate which they tentatively identified as adenylosuccinic acid, already familiar as an intermediate of adenine nucleotide synthesis in yeast (Carter and Cohen, 1955). Two enzymes are involved in the conversion of IMP to AMP: adenylosuccinate synthetase and adenylosuccinate lyase. The synthetase requires aspartate as amino donor, and GTP provides the energy for the reaction (Davey, 1961). In aerobic skeletal muscle resynthesis of AMP follows deamination in a cyclic process; this has been termed the purine nucleotide cycle and has been studied in detail in rat muscle homogenates (Lowenstein, 1971, 1972; Tornheim and Lowenstein, 1972). The cycle is shown diagrammatically in Fig 1.1. It may function in the regulation of the ammonia concentration in the cell, or in the deamination of amino acids which can transfer their amino groups ultimately to aspartate (Lowenstein, 1972). Its suggested relationship with energy metabolism is outlined in section 1.4 below.

AMP is not the only precursor of ammonia in muscles. Certain insect muscles reportedly lack AMP deaminase (Gilmour and Calaby, 1953; Gerez and Kirsten, 1965), yet they still produce ammonia

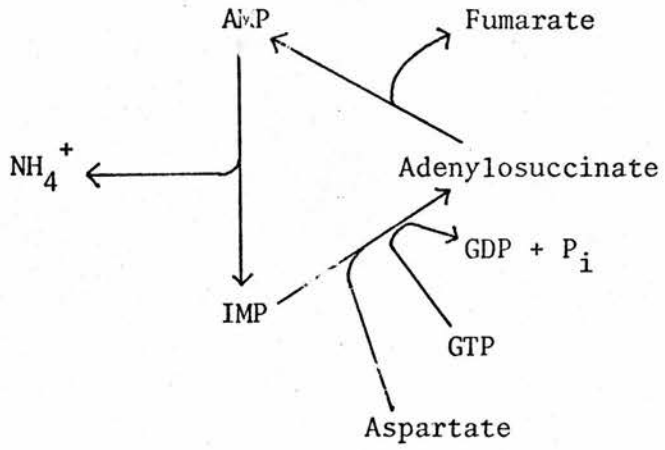


Fig 1.1. The Purine Nucleotide Cycle (after Lowenstein, 1972).

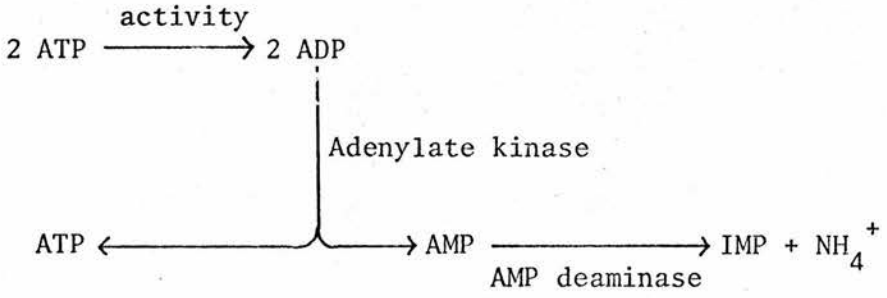


Fig 1.2. The actions of adenylate kinase and AMP deaminase in working skeletal muscle.

Tissue	AMP-deaminase activity µmoles/g fresh wt./min After Lowenstein (1972).	Ammonia concentration µmoles/g fresh wt. (Chapter 3, Table 3.1).
Rat leg muscle	127	0.410
Rat heart muscle	1.6 - 4.4	0.430

Table 1.1 Showing the lack of correlation between AMP-deaminase levels and ammonia concentration in skeletal and cardiac muscle.

(Gerez and Kirsten, 1965). Furthermore, large differences in AMP deaminase activity between tissues, for example between rat heart and skeletal muscle (Lowenstein, 1972), are not paralleled by differences in tissue ammonia concentration (Table 1.1). In the hypoxic heart, the deamination of adenosine occurs in preference to the deamination of AMP (Gerlach et al., 1963; Richman and Wyborny, 1964; Baer et al., 1966), but this is not the source of the ammonia produced by adequately oxygenated heart (see Chapter 5, Section 5.1).

Glutamine has been established as the precursor of ammonia in the kidney (Van Slyke et al., 1943; Pitts et al., 1965) and as a storage form of amino nitrogen (Braunstein, 1947). Decreases in the glutamine content of perfused rat heart (Hicks and Kerly, 1960; Ottaway, 1969a) and guinea pig heart (Gailis and Benmouyal, 1973) have been observed, and have been attributed to the action of glutaminase (Ottaway, 1969b). Glutamine is thus a possible precursor of ammonia in heart.

Deamination of glutamate could in theory give rise to ammonia. In the mitochondria, where the $NAD^+/NADH$ ratio is much lower than in the cytoplasm (Veech et al., 1969), the equilibrium constant of glutamate dehydrogenase (Frieden, 1963; Bergmeyer, 1965) suggests that this reaction is of greater importance in the fixation of free ammonia than in its production. Nevertheless, some form of compartmentation may be required to keep the ammonia concentration low in the neighbourhood of glutamate dehydrogenase (see below, Section 1.3), and under these conditions this enzyme could catalyze a slow production of ammonia from glutamate. The possibility of intra-mitochondrial compartmentation with respect to glutamate metabolism

has been discussed by Tager (1966).

The massive release of ammonia on trauma or hypoxia (Parnas and Mozolowsky, 1927) must be distinguished from the normal synthesis of ammonia under physiological conditions. Traumatic ammonia production in all tissues results from the degradation of adenine nucleotides. Parnas believed that breakdown of the muscle structure destroyed some form of intracellular compartmentation which had hitherto rendered AMP inaccessible to its deaminase (Parnas, 1935). It is equally possible that trauma damages the oxygen supply to the muscle. The low intracellular oxygen tension reduces the activity of the electron transport chain which is responsible for the oxidative phosphorylation of ADP. Under these conditions the requirement for ATP is met by the action of adenylate kinase, which catalyzes the dismutation of ADP to produce ATP and AMP:

$$2\ ADP \longrightarrow ATP + AMP$$

The yield of ATP is increased if AMP is removed by deamination; this results in the "traumatic" ammonia release. Since the concentration of GTP is also reduced in hypoxia (Gerlach et al., 1963) the reamination of IMP by aspartate is inhibited (Lowenstein, 1971, 1972). In contrast, under physiological conditions the precursor of ammonia differs between tissues: in skeletal muscle it may be AMP; in other tissues, ammonia may derive from some other amino compound, such as glutamate or glutamine.

1.2 The Metabolic Fate of Ammonia in Muscle.

The question whether the converse process, namely ammonia

fixation, took place in skeletal muscle was the subject of fierce argument in the early literature. Parnas believed that ammonia, once formed, was never re-utilized by the muscle, but was carried away by the bloodstream (Parnas, 1930, 1935; Parnas and Lewinsky, 1935). His results were supported more recently by those of Chiosa and Busneag (1971), who failed to show any utilization of ammonia by rat leg muscle homogenates. However, a substantial body of evidence suggested that ammonia produced by active muscles, namely frog muscle (Embden and Wassermeyer, 1928a; Embden et al., 1928b), rat leg muscle (Gerez and Kirsten, 1965) and a rat heart-lung preparation (Cruickshank and McClure, 1936), could be re-used during the recovery of the muscle.

Three systems are known to be concerned with ammonia fixation in vertebrate metabolism: glutamate dehydrogenase, glutamine synthetase and carbamyl phosphate synthetase (Langendorf, 1969). The equilibrium constant of glutamate dehydrogenase renders it suitable for the assimilation of ammonia into glutamate:

$$NH_4^+ + \alpha OG + NADH \longrightarrow H_2O + Glu + NAD^+$$

This reaction is available in all tissues which possess glutamate dehydrogenase; the enzyme is more active in heart than it is in skeletal muscle (Lowenstein, 1972). The hydrogen donor can be isocitrate, succinate or malate (Tager, 1966); or it can be α-oxoglutarate itself, by a dismutation process (Krebs and Cohen, 1939; Hunter and Hixon, 1949; Worcel and Erecinska, 1962). The coenzyme may be NADPH rather than NADH (Tager, 1965). If this reaction were allowed to proceed uncontrolled the high ammonia

concentration in muscle would soon lead to depletion of α-oxoglutarate and failure of energy metabolism in the tissue. The necessity for control of the reaction at a low rate, possibly by means of permeability barriers limiting access of the ammonia to the enzyme, suggests that it can only operate slowly in the fixation of ammonia.

Glutamine synthesis is only available as a method of ammonia fixation in tissues that possess glutamine synthetase. This enzyme, though present in avian heart (Gothoskar et al., 1960; Wu, 1963; Patel and Ramakrishnan, 1969) has only once been reported in rat heart (Trush, 1963b); other attempts to measure it directly in mammalian heart have failed (Wu, 1963; Iqbal and Ottaway, 1970), although similar techniques showed that the enzyme was present in rat skeletal muscle (Iqbal and Ottaway, 1970).

Less direct evidence for the occurrence of glutamine synthetase in muscle was provided by experiments in which ammonia administration caused an increase in tissue glutamine concentration. Raising the blood ammonia concentration (by ammonia infusion or by hepatectomy) increased the glutamine content of dog skeletal muscles (Flock et al., 1953; Stabenau et al., 1959) and rat heart (Tigerman and McVicar, 1951; Kato, 1968) and increased the output of glutamine by skeletal muscle (Hills et al., 1972; Ruderman and Lund, 1972). Administration of ^{15}N-labelled ammonium ion to live animals led to the recovery of isotope in glutamine of skeletal muscle (Duda and Handler, 1958; Silakova and Trush, 1962; Ferdman et al., 1963) and heart (Duda and Handler, 1958; Ferdman et al., 1963), glutamine was more highly labelled in heart than in any other tissue studied (Duda and Handler, 1958). This is not in itself a conclusive demonstration

that glutamine synthetase occurs in heart or muscle. These tissues could take up from the blood glutamine synthesized by other organs such as brain (Flock et al., 1953; Clark and Eiseman, 1958; Monder, 1965), or liver (Lueck and Miller, 1970), or even by gut bacteria (Kosharov et al., 1967). The demonstration of glutamine synthetase is much more conclusive if glutamine production can be localized by a suitable technique to a particular organ, such as human heart (Keul et al., 1964, 1966). It is even more convincing if an isolated tissue or organ preparation is used. Rabbit heart slices produced glutamine in response to added ammonia (Trush, 1963a). Synthesis of labelled glutamine from ^{14}C-glutamate occurred in perfused rabbit heart (Doell and Felts, 1959) and perfused guinea pig heart (Asano, 1968). Perfused guinea pig heart also synthesized ^{14}C-glutamine from other labelled precursors: glucose (Davis and Quastel, 1964; Gailis and Benmouyal, 1973), lactate (Davis and Quastel, 1964), acetate and pyruvate (Asano, 1968; Watanabe, 1968; Gailis and Benmouyal, 1973). Incorporation of isotope from acetate and pyruvate into glutamine was increased when ammonia was also added to the perfusing fluid (Watanabe, 1968), Asano (1968) however found that ammonia increased the incorporation of label from pyruvate, but not from acetate.

These experiments provide strong evidence that glutamine can be synthesized by the human, rabbit or guinea pig hearts studied. Attempts made in this laboratory to reproduce the experiments of Trush (1963b) did not demonstrate any glutamine synthesis in rat heart (Iqbal and Ottaway, 1970). The existence of glutamine synthetase in rat heart thus remains in some doubt.

1.3 Ammonia Toxicity.

The concentration of ammonia in muscle is high (Chapter 3, Table 3.1). Three problems associated with the high concentration are immediately raised. Firstly, ammonia is often regarded as a toxic substance. Secondly, does the high concentration serve any function in the cell? Thirdly, what mechanisms are responsible for maintaining a concentration which is so much higher than that in plasma?

Failure of urea synthesis, as in hepatic failure, leads to an increase in blood ammonia (Bessman and Bessman, 1955; Fenton, 1962; Williams et al., 1972) which may be one of the causes of cerebral symptoms found in such cases (Fenton et al., 1966). Ammonia decreases the aerobic metabolism of carbohydrates in brain, even though the rate of glycolysis may be increased (James et al., 1972). The increase in the proportion of glucose metabolized anaerobically implies a lesion either in the Krebs cycle or in the electron-transport system. It is usually held that ammonia combines with α-oxoglutarate to form glutamate, with consequent depletion of α-oxoglutarate and other Krebs cycle intermediates in the cell (Bessman and Bessman, 1955; Worcel and Erecinska, 1962; Chow et al., 1970; Clifford et al., 1972). Further conversion of glutamate to glutamine may exacerbate this effect, particularly in brain (Clark and Eiseman, 1958; Warren and Schenker, 1964; Williams et al., 1972). The brain is thus peculiarly susceptible to ammonia poisoning, and convulsions usually accompany ammonia intoxication. Alternatively, the primary cause of the decreased oxidative metabolism may be a depletion of NADH and consequent

impairment of cellular respiration. Glutamate dehydrogenase may compete with the respiratory chain for NADH (Worcel and Erecinska, 1962), or there may be a specific activation by ammonia of NADH oxidases (Katunuma et al., 1966).

Ammonia forms complexes with certain metabolically important inorganic ions. The formation of magnesium ammonium phosphate and calcium ammonium phosphate has been suggested. These compounds are treated as waste products by the body, and are excreted in the urine (Chow et al., 1971). Thus ammonia interferes with the metabolism of calcium, magnesium and phosphate in the body: it hinders the absorption of calcium and phosphate from the duodenum, and decreases the incorporation of these ions into bone (Chow et al., 1971, 1972).

It has been proposed that blood ammonia is maintained at a low level by the action of glutamine synthetase in skeletal muscle (Bessman and Bessman, 1955; Bessman and Bradley, 1955; Langendorf, 1969). The disorder of familial protein intolerance, in which dietary amino acids and proteins cause a rise in blood ammonia not seen in normal individuals, may involve a lesion in glutamine metabolism (Malmquist et al., 1971). The function of these detoxification mechanisms is to prevent the toxic effects of ammonia on the brain (Bessman and Bessman, 1955).

Both skeletal and cardiac muscles possess glutamate dehydrogenase (Lowenstein, 1972) and its equilibrium constant (Frieden, 1963; Bergmeyer, 1965) suggests that even small amounts of ammonia would be removed by combination with α-oxoglutarate. One may wonder therefore why the high concentration of ammonia which exists in muscle (Chapter 3, Table 3.1) is not toxic to the tissue. The most logical explanation is to assume that permeability barriers

in the cell prevent the bulk of the ammonia from reaching the glutamate dehydrogenase. The ammonia concentration in the neighbourhood of the enzyme would thus be kept low and fixation of ammonia by glutamate dehydrogenase would be slow.

1.4 Ammonia and Energy Metabolism.

Ammonia is produced continuously by resting muscle (Parnas, 1935). Production is increased by trauma (Parnas and Mozolowsky, 1927; Embden et al., 1928a), death (Bendall and Davey, 1957), and by increasing the ambient temperature of cold-blooded animals (Embden and Wassermeyer, 1928a; Parnas and Lewinsky, 1935). The increase of greatest importance to homeothermic animals occurs in response to muscular activity, but has been observed in frog leg muscle (Parnas and Mozolowsky, 1927; Embden et al., 1928a; Busneag and Chiosa, 1971), and insect muscle (Gerez and Kirsten, 1965), as well as in rat leg muscle (Brown et al., 1957; Gerez and Kirsten, 1965; Chiosa and Busneag, 1971), dog leg muscle (Duchêne-Marullaz et al., 1964a), human forearm (Parnas et al., 1927; Allen and Conn, 1960; Duchêne-Marullaz et al., 1964a), rabbit heart (Feinberg and Alma, 1960; Kato, 1968; Watanabe, 1968; Watanabe et al., 1969), dog heart (Duchêne-Marullaz et al., 1966; Kobayashi, 1967) and human heart (Keul et al., 1966). Increases in blood ammonia due to general muscular activity have been noted in dogs (Schwartz et al., 1958; Duchêne-Marullaz et al., 1964b) and in humans (Schwartz et al., 1958; Keul et al., 1964). Despite the increase in ammonia production during activity, early investigators realized that ammonia formation had no direct connection with the process of muscular contraction.

Ammonia produced by frog muscle showed no correlation with lactate produced during activity (Embden and Wassermeyer, 1928a). Similar amounts of exercise resulted in different rates of ammonia production under different conditions (Parnas and Lewinsky, 1935). Leg muscles of rats accustomed to regular bouts of strenuous activity showed no increase in ammonia output on stimulation (Chiosa and Busneag, 1972).

Parnas saw the function of adenylate deaminase in skeletal muscle as a means of destroying AMP formed during periods of muscular activity, believing that excess free AMP could exert harmful effects on the muscle and on neighbouring tissue, whereas IMP was harmless (Parnas, 1935). Modern views are summarized by Lowenstein (1971, 1972). Muscular activity produces ADP. In strenuous activity, oxidative phosphorylation of ADP may be insufficient to keep pace with ATP demands. Under these conditions, as in hypoxia, the ATP requirement is met by adenylate kinase, which catalyzes the production of ATP and AMP from ADP. The function of AMP deaminase is to remove AMP, upsetting the adenylate kinase equilibrium and making more ATP available for the short-term needs of the muscle (Fig 1.2). This cannot continue for long periods without severely depleting the muscle's adenine nucleotide reserves. Resynthesis of AMP and ATP from IMP must therefore occur during the recovery of the muscle.

Some insect muscles lack adenylate deaminase (Gilmour and Calaby, 1953; Gerez and Kirsten, 1965); other muscles, such as rat heart, possess the enzyme in much lower activity (Lowenstein, 1972). In fact it is unlikely that the Lowenstein purine nucleotide cycle is of great metabolic importance in rat heart (see Chapter 5,

Section 5.1). Such muscles do not differ greatly from skeletal muscle in respect of their ammonia concentration (Chapter 3, Table 3.1). It is probable therefore that ammonia has a distinct function in the field of muscle metabolism, other than being merely a by-product of the purine nucleotide cycle. Its function may include enzyme regulation (Lowenstein, 1972).

1.5 Ammonia and Enzyme Activity.

Experiments in vitro have shown that ammonia has an activating effect on the membrane sodium and potassium transporting ATPase of erythrocytes (Post et al., 1960) and kidney (Klahr et al., 1970), on heart myosin ATPase (Hjalmarson et al., 1970; Rovetto et al., 1972), on lipoprotein lipase of rat heart (Korn and Quigley, 1955; Whayne and Felts, 1971), and on fructose diphosphatase (Black et al., 1972), pyruvate kinase (Kayne, 1971) and AMP deaminase (Ashman and Atwell, 1972) from rabbit muscle. However, the ammonia concentrations required to produce activation are always far in excess of that found in the cell, and the effect of ammonia is likely to be insignificant in comparison with the effects of other intracellular cations such as potassium.

However, physiological concentrations of ammonia may have a regulatory effect in decreasing the flux through the Krebs cycle (Katunuma et al., 1966), increasing flux through the pentose phosphate shunt in yeast (Holzer and Witt, 1960) and in erythrocytes (de Loecker, 1964), and increasing the rate of Embden-Meyerhof glycolysis in yeast (Holzer and Witt, 1958), E. coli (Holzer and Grunicke, 1961), erythrocytes (de Loecker, 1964; Kloppick et al.,

1967) and brain (James et al., 1972). The most detailed investigation of the activation of a specific enzyme by ammonia concerns phosphofructokinase. The acceleration by ammonia of erythrocyte glycolysis was traced to an effect on this enzyme (Kloppick et al., 1967). Phosphofructokinase from mouse brain (Passonneau and Lowry, 1964), rabbit liver (Kemp, 1971) and rabbit muscle (Abrahams and Younathan, 1971) is activated by ammonia. Half-maximal activation of the rabbit muscle enzyme occurs around the physiological ammonia concentration (Chapter 3) of 0.3 m mol/l (Abrahams and Younathan, 1971); thus changes in muscle ammonia concentration around this value should have a profound effect on the rate of glycolysis, although the effects of other proposed phosphofructokinase effectors must not be disregarded (Randle et al., 1968). An increase in ammonia concentration on muscular activity would cause an increase in the rate of glycolysis: hence the change in ammonia concentration on activity reinforces the effects of changes in ATP, ADP, AMP and inorganic phosphate in the mediation of the Pasteur effect.

1.6 Transport of Ammonia across Membranes.

Transport of ammonia across cell membranes has been a subject of contention in the literature for many years. Ammonia crosses most membranes rapidly, but it is by no means certain whether the permeant species is unionized ammonia (NH_3) (Cooper and Osterhout, 1930; Jacobs and Parpart, 1938; Jacobs. 1940) or ammonium ion (Fenn et al., 1945).

The proportion of ammonia in the uncharged form depends on

the equilibrium: $NH_4^+ \rightleftharpoons NH_3 + H^+$, which depends on the pH of the solution. Although the pK_a for the dissociation of NH_4^+ is relatively unaffected by the presence of other solutes, it is markedly temperature-dependent (Bank and Schwartz, 1960); under physiological conditions it is 9.03 (Klocke et al., 1972). Changes in pH around the "physiological" value of 7.4 will thus cause large variations in the NH_3 concentration, without greatly affecting the NH_4^+ concentration, since at pH 7.4 the ratio of NH_4^+ to NH_3 is 43 to 1. Proof that NH_3 is the permeant species has rested largely on the demonstration of a pH-dependent distribution of ammonia across membranes. NH_3 diffuses from the more alkaline side of a membrane, where its concentration is higher, to the more acid side. Equilibrium is reached when the NH_3 concentration on both sides is the same. The NH_4^+ concentration at equilibrium is greater on the acid side. The passage of NH_3 has the effect of rendering the acid side more alkaline: diffusion of NH_3 in one direction is equivalent to the passage of protons in the opposite direction (see Fig 1.3; see also Henderson, 1971). This phenomenon could easily be mistaken for direct exchange of NH_4^+ for H^+. It leads to dissipation of the original pH gradient unless H^+ is returned, possibly in exchange for Na^+ or some other cation (Fig 1.4), by a carrier mechanism. The overall picture could be mistaken for a direct exchange of NH_4^+ for Na^+, except that in the former case the primary event depends on pH rather than on Na^+ concentration.

Cooper and Osterhout (1930) described a pH-dependent ammonia equilibrium in cells of Valonia macrophysa. The entry of NH_3 caused alkalization of the interior, and was accompanied by K^+ efflux. Similarly, human erythrocytes were permeable to NH_3

(Jacobs and Parpart, 1938; Klocke et al., 1972) and a mathematical prediction of the pH-dependent distribution was possible (Jacobs, 1940). Stabenau and co-workers (1959) observed a pH-dependent equilibrium indicative of NH_3 diffusion between blood and muscle, blood and brain and blood and cerebrospinal fluid in dogs. Bessman et al., (1961) found on the other hand that the uptake of ammonia from blood depended solely on the arterial concentration, and not on pH. The pH-dependent diffusion of NH_3 has been demonstrated in sheep rumen (Mooney and O'Donovan, 1970), rat intestine (Swales et al., 1970) and human colon (Castell and Moore, 1971), though not in hamster ileum (Mossberg, 1967). Langendorf (1969) showed that the toxicity of ammonium salts increased with their alkalinity. NH_3 diffusion also occurs in kidney tubules (White et al., 1973). NH_3 diffusion has also been postulated for ammonia uptake by chloroplasts (Crofts, 1967; Schuldiner and Avron, 1971) and mitochondria (Chappell and Haarhoff, 1967; Papa et al., 1969; Van Dam and Meyer, 1971). The necessity for concomitant proton movements is obviated if a weak acid crosses the membrane at the same time, the membrane being permeable to the undissociated form of the weak acid (Fig 1.5). Thus the uptake of ammonium acetate is faster than the uptake of ammonium chloride, for example in rat brain synaptosomes (Keen and White, 1971).

As indicated above (see Fig 1.4), pH-dependent NH_3 diffusion can be accompanied by cation movements. Ammonia uptake accompanied by egress of cations, as described by Albano and Francavilla (1971) in rat erythrocytes, is therefore not in itself a proof of NH_4^+ transport. On the other hand, non-dependence of the ammonia distribution on pH, or ammonia transport when the pH-gradient is

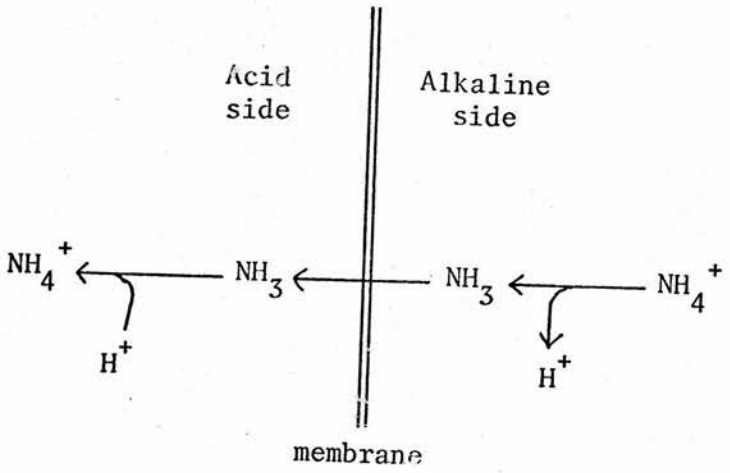


Fig 1.3. The diffusion of NH_3 from right to left is equivalent to the passage of protons from left to right.

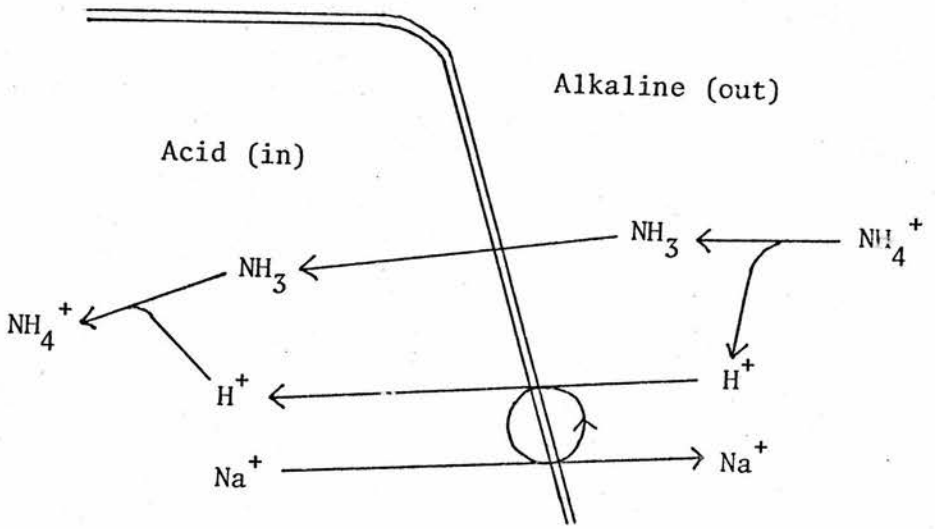


Fig 1.4. Exchange of protons for sodium ions. Overall events are equivalent to an exchange of ammonium for sodium.

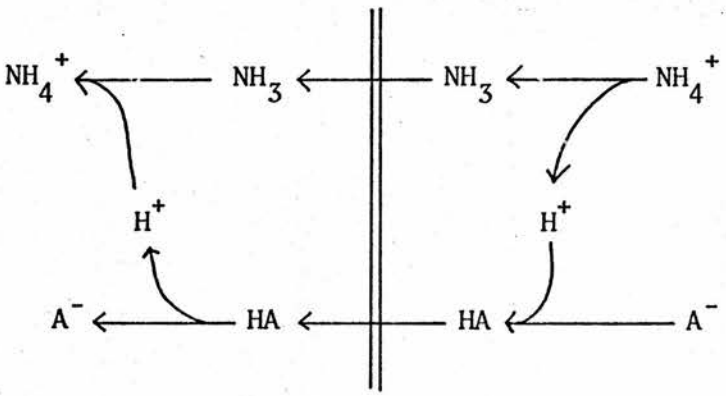


Fig. 1.5. Diffusion of ammonia is accelerated by the presence of the weak acid anion, A^-.

non-existent or operating in the reverse direction, does suggest that the permeant species is not uncharged ammonia.

Fenn et al., (1945) found that the uptake of ammonia by frog muscle was accompanied by an efflux of K^+. Freshwater animals require to excrete a large quantity of ammonia yet need to maintain osmotic balance. It has been proposed that ammonium ion is exchanged for Na^+ or possibly H^+ by frogs (Garcia-Romeu, 1971) and freshwater fish (Maetz and Garcia-Romeu, 1964; Kerstetter et al., 1970). The effect of pH was not investigated. Excretion of ammonia by toad bladder is independent of pH (Frazier and Vanatta, 1971), though it is also unaffected by abolition of the trans-membrane potential difference, which would also seem to rule out simple electrophoretic diffusion of NH_4^+. Simple electrophoretic diffusion of NH_4^+ across the human colon from a positive serosal to a negative mucosal surface was claimed by Wolpert et al., (1970). This is at variance with the results of Castell and Moore (1971) cited above, although the passage of ammonia in Wolpert's experiments is in the opposite direction and may well require a different mechanism.

Most of the proposed mechanisms involve a passive distribution of ammonium ion according to a pre-existing Nernst equilibrium, controlled mainly by the K^+ distribution. On the other hand NH_4^+ transport can be the primary event, as suggested by the finding of a specific NH_4^+ permease in a Penicillium species (Hackette et al., 1970). A temperature-dependent NH_4^+-concentrating system, possibly a permease, has been described in an alga (Pribil and Kotyk, 1970). The uptake of NH_4^+ against a concentration gradient requires energy. Post and Jolly (1957) have shown that NH_4^+ can be transported into human erythrocytes by the Na^+/K^+-dependent ATPase, in place of K^+;

external NH_4^+ can also substitute for K^+ in the activation of Na^+ efflux. It cannot however replace sodium. Activation of ATPase by ammonium ion can be shown in purified preparations of the enzyme (Post et al., 1960; Post and Sen, 1967). Sachs (1967) however suggested that NH_4^+ could bind to the ATPase and inhibit K^+ influx but could not be carried by it, ammonia entering the cells only in the uncharged NH_3 form. Na^+/K^+ transport ATPases of beef heart (Yoda and Hokin, 1972) and rabbit kidney (Klahr et al., 1970) can also bind ammonium ion.

Our understanding of ammonia transport is therefore by no means complete. There seems to be a general pattern of NH_4^+ diffusion in lower organisms and of NH_3 diffusion in higher animals. The transport of ammonia across the membranes of mammalian muscle has never been investigated in detail, and no studies have been undertaken in heart muscle.

1.7 Amino Acid Metabolism in Heart Muscle.

Amino acids in the intracellular pool of heart muscle derive both from plasma and from the breakdown of cellular proteins. The concentration of free amino acids in rat heart has been measured by several workers (Manchester and Wool, 1963; Scharff and Wool, 1965a; Herbert et al., 1966; Kuttner and Lorincz, 1969; Morgan et al., 1971). Their results are summarised in Table 5.5 of Chapter 5. Many of these amino acids can be formed or degraded within the heart by transamination with glutamate or other amino acids. Cammarata and Cohen (1950) list those amino acids which can transaminate with glutamate in heart. Those which cannot be metabolized in this way are gained

and lost solely by passage across the membrane, and by synthesis or degradation of proteins. Transport of amino acids across the membrane is an active process and is insulin-dependent (Scharff and Wool, 1965a,b, 1966; Manchester, 1970).

The role of amino acids in protein synthesis in muscle is well documented (see Manchester and Young, 1959; Manchester and Wool, 1963; Morgan et al., 1971). Incorporation of amino acids into protein is increased by insulin (Manchester and Young, 1959, 1961; Manchester and Wool, 1963; London, 1972). It is possible that protein synthesis is the function of a small, metabolically active fraction of the total tissue free amino acids, at least in skeletal muscle (Hider et al., 1969, 1971) and diaphragm (Kipnis et al., 1961); this pool is in rapid equilibrium with the ECF and it may be located in the T-tubules or in the sarcoplasmic reticulum (Hider et al., 1971).

The oxidation of amino acids provides a relatively small fraction of the heart's energy requirements under normal circumstances (Keul et al., 1964, 1965, 1966). This fraction probably increases when other substrates are in short supply (Clarke, 1957), as in starvation. Oxidation of amino acids involves the detachment of the amino group from the carbon skeleton by transamination, followed by the oxidation of the resultant oxo-acid to yield energy. Thus oxidation is limited to those amino acids for which transaminases occur (Cammarata and Cohen, 1950) and whose corresponding oxo-acids can be metabolized by the heart. Quantitatively the most important are those which are present in the highest concentration, and which are directly related to intermediates of energy metabolism, namely alanine, glutamine, aspartate and glutamate. Glycine (Lorber and Olsen, 1946; Coulson and Hernandez, 1968) and lysine (Felig and

Wahren, 1971; Felig, 1973) are not oxidized by the heart, although they are also present in relatively high concentration.

Glutamate is the direct product of the transamination of amino acids with α-oxoglutarate, and of the hydrolysis of glutamine by glutaminase (Ottaway, 1969a,b). However, there is no net output of glutamate by the heart (Keul et al., 1966) and it must therefore be metabolized further in this tissue. Oxidative deamination catalyzed by glutamate dehydrogenase produces α-oxoglutarate and ammonia. This reaction has already been discussed (p. 8). Alternatively, reaction of the glutamate with pyruvate produced in glycolysis yields α-oxoglutarate and alanine. Studies have shown that both ammonia (Feinberg and Alma, 1960; Keul et al., 1964, 1966) and alanine (Carlsten et al., 1961; Ottaway, 1969a) are produced by heart. These represent mechanisms for ridding the tissue of unwanted amino nitrogen without involving such a serious loss of carbon skeletons as would be the case if glutamate itself were released. There is also a net output of glutamine in hearts of some species (see Keul et al., 1964). The interpretation of this as a method for disposal of amino nitrogen is less clear as there is some doubt about the existence of glutamine synthetase in rat heart (Trush, 1963b; Iqbal and Ottaway, 1970). This aspect of glutamine metabolism has already been discussed (p. 11).

Heart takes up glutamate and glucose, and releases alanine and ammonia into the plasma. Skeletal muscle displays a similar amino acid balance. This phenomenon has led some workers to postulate an "alanine cycle" (see Felig, 1973, for a review). The cycle is completed in the liver, where alanine is converted to glucose by gluconeogenesis (Ishikawa et al., 1972) and the nitrogen is released into the plasma as glutamate or disposed of as urea. This cycle will

be discussed in greater detail in Chapter 5 (Section 5.3.3) where a review of the literature will be presented.

1.8 The Structure of Cardiac Muscle.

Cardiac muscle differs from skeletal muscle in a number of respects. The fibre orientation is more haphazard, and the cross-striation of the fibrils is less regular (see Chapter 6). The structure of cardiac muscle is described in detail by Bloom and Fawcett (1968), pp. 285-297, where a diagram is presented. Fundamentally the tissue consists, as does skeletal muscle, of actomyosin fibres embedded in an aqueous sarcoplasm containing enzymes, substrates and other essential substances. Mitochondria fill the interfibrillar spaces in a columnar pattern (Klingenberg, 1964). There are more mitochondria in cardiac than in skeletal muscle, and they are larger (Klingenberg, op. cit.); this reflects the importance to cardiac muscle of a constant energy supply. The complete fibre is surrounded by a membrane, the sarcolemma, which has two properties. Firstly, it is excitable and can carry an electrical impulse along the fibre from the motor nerve ending. Secondly, it is a semipermeable membrane which regulates the passage of substrates, cofactors and other small molecules into the sarcoplasm.

The heart muscle syncytium is large in comparison with most cells of the body. Thus both functions of the membrane are rendered more efficient by prolongations into the sarcoplasm which increase the surface area of the membrane. These form two distinct tubular systems in both skeletal and cardiac muscle which are known as the transverse (T-) tubules and the longitudinal (L-) tubules or

sarcoplasmic reticulum (Huxley, 1964). The T-tubules originate as invaginations of the sarcolemma (Ishikawa, 1968). Extracellular ferritin diffuses readily into the T-tubules of skeletal muscle (Huxley, 1964; Ishikawa, 1968) and cardiac muscle (Forssmann and Girardier, 1966), where it can be seen in electron-micrographed sections. Horseradish peroxidase also enters the T-tubules of rat heart muscle (Forssmann and Girardier, 1970) where it can be localized by staining with diaminobenzidine. These experiments suggest that the lumen of the T-tubules is continuous with the extracellular space. The apertures of the T-tubules on the surface of the sarcolemma have been visualized in guinea pig heart muscle by a freeze-etching technique (Rayns and Simpson, 1967).

The T- and L-tubules however are not themselves continuous; neither ferritin (Huxley, 1964; Forssmann and Girardier, 1966; Ishikawa, 1968) nor horseradish peroxidase (Forssmann and Girardier, 1970) can enter the L-tubules of heart or skeletal muscle. However, there is evidence that small molecules in the ECF such as sucrose can penetrate the L-system and cause swelling (Birks and Davey, 1969). Thus at certain points the T- and L-systems are in contact, but the lumina of the two systems are distinct. These juxtapositions or triads are very regular in skeletal muscle (Kelly, 1969); the T-system forms a flattened sac flanked on either side by a terminal lacuna of the L-system. In cardiac muscle similar connections exist but they are less regular in appearance (Forssmann and Girardier, 1966). The T-tubules are tortuous and can make contact with the L-system at any point (Forssmann and Girardier, 1970). The L-system tubules appear to be continuous with the outer mitochondrial membrane (Forssmann and Girardier, 1966). Postulated functions of the T- and

L-systems are firstly, transmission of the electrical impulse from the nerve ending throughout the fibre so that all the fibrils contract in phase (Huxley, 1964; Forssmann and Girardier, 1966, 1970) and secondly, conveyance of substrates such as free fatty acids from the ECF directly to the mitochondria to maintain the energy supply required for continual contraction (Forssmann and Girardier, 1966, 1970). These membrane systems will be considered in relation to the handling of ammonia and amino acids by the tissue (Chapter 3; Chapter 7).

Conclusion.

It is clear from the foregoing presentation that there is a need for a study of the metabolism of ammonia in muscle tissues. The investigation to be reported in the following pages shows that ammonia is produced by the isolated perfused rat heart, and attempts to clarify some aspects of its metabolism in this organ; several fields are indicated into which further research ought to be directed.

CHAPTER 2

MATERIALS AND METHODS

Chapter 2. Materials and Methods.

2.1 Materials.

2.1.1 Laboratory Chemicals

All inorganic chemicals used in research in Edinburgh were products of B.D.H. Biochemicals Ltd., Poole, Dorset, England. Those used in enzyme location studies in Konstanz were products of E. Merck, Darmstadt, W. Germany. All were analytical grade. The following organic chemicals were also obtained from B.D.H. Biochemicals: Glucose, o-dianisidine dihydrochloride, INT, Phenol, disodium EDTA, ninhydrin, hydrindantin, nitron, glycerol, glycine, aspartic acid, monosodium glutamate, alanine and glutamine. The following were products of Sigma (London) Ltd: phenazine methosulphate, nitro-blue tetrazolium, diaminobenzidine tetrahydrochloride, agarose, glycerol-gelatine, D-sorbitol and dimethyloxazolidinedione. α-oxoglutarate was obtained from Boehringer and Soehne GmbH, Mannheim, W. Germany, as were sodium lactate, sodium succinate, aspartic acid and monosodium glutamate used as substrates in histochemical enzyme localization. Bovine serum albumin was a product of Armour Pharmaceutical Co. Ltd., Eastbourne, England. ^{14}C-sorbitol was obtained from the Radiochemical Company Ltd., Amersham, England and ^{15}N-ammonium chloride from Prochem (The British Oxygen Co. Ltd.). Dioxane-based scintillant for measurement of ^{14}C-sorbitol was a product of Koch-Light Laboratories Ltd, Colnbrook, Bucks, England. Analytical grade diethyl ether was obtained from McFarlane Smith Ltd, Edinburgh. Glass-distilled water was used throughout the study.

2.1.2 Fine Chemicals and Enzymes

NAD^+, $NADP^+$, the disodium salts of NADH and ADP, and cytochrome c were obtained from Boehringer and Soehne, as were all enzymes, with the following exceptions: lyophilized alcohol dehydrogenase was obtained from B.D.H. Biochemicals; glucose oxidase ("Fermcozyme") and peroxidase from Hughes and Hughes Ltd, Romford, Essex, England; and catalase from Koch-Light Laboratories. All enzymes from Boehringer and Soehne were bought as crystalline suspensions in ammonium sulphate, with the exception of glutamate dehydrogenase, which was a solution in 50% glycerol.

2.1.3 Abbreviations

The following abbreviations are used in the text:

ADH	alcohol dehydrogenase
ADP	adenosine-5'-diphosphate
DAB	3,3'-diaminobenzidine
DMO	5,5-dimethyl-2,4-oxazolidinedione
ECF	extracellular fluid
EDTA	ethylenediamine tetraacetic acid
Glu DH	glutamate dehydrogenase
GOT	glutamate oxaloacetate transaminase
GPT	glutamate pyruvate transaminase
ICF	intracellular fluid
INT	2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride
LDH	lactate dehydrogenase
MDH	malate dehydrogenase

$NAD(P)^+$	nicotinamide adenine dinucleotide (phosphate)
NAD(P)H	reduced $NAD(P)^+$
Nitro-BT	nitro-blue tetrazolium
NH_3	undissociated ammonia
NH_4^+	ammonium ion
NRM	ninhydrin-reactive material
∝OG	∝-oxoglutarate
PMS	phenazine methosulphate
SDH	succinate dehydrogenase
TEA	triethanolamine

2.2 Metabolite Assays.

Specific optical assays which involved the enzymic production or utilization of NADH were carried out in 1cm glass cuvettes, test volume 1.0 ml, at 37°C. The change in absorbance at 340 nm was monitored continuously by an SP 800 spectrophotometer (Pye-Unicam, Cambridge). An SP 825 cuvette changer enabled four reactions to be carried out simultaneously, and an SP 850 scale expander connected to a Servoscribe recorder provided a record of absorbance changes. In all cases the reaction was started by adding enzyme to the mixture of the other components pre-warmed to 37°C. The Berthelot method for ammonia estimation (Section 2.2.7), the tetrazolium method for glutamate assay (Section 2.2.8.1), and the assays for glucose and total ninhydrin-reactive material (Sections 2.2.1 and 2.2.4) required absorbance measurement at the end of a specified period and comparison with a standard curve. Absorbance was measured at the appropriate wavelength in a Coleman Junior II Spectrophotometer.

2.2.1 Glucose

Glucose was assayed by the method of Huggett and Nixon (1957), using glucose oxidase ("Fermcozyme") and peroxidase with dianisidine as the colour reagent. Absorbance at 440 nm was linearly related to concentration from 0 to 0.3 μ moles of glucose per assay (Fig 2.1).

2.2.2 Lactate

Lactate was assayed using lactate dehydrogenase and NAD^+ in glycine-hydrazine buffer at pH 9.0 (Hohorst, 1965). The increase in

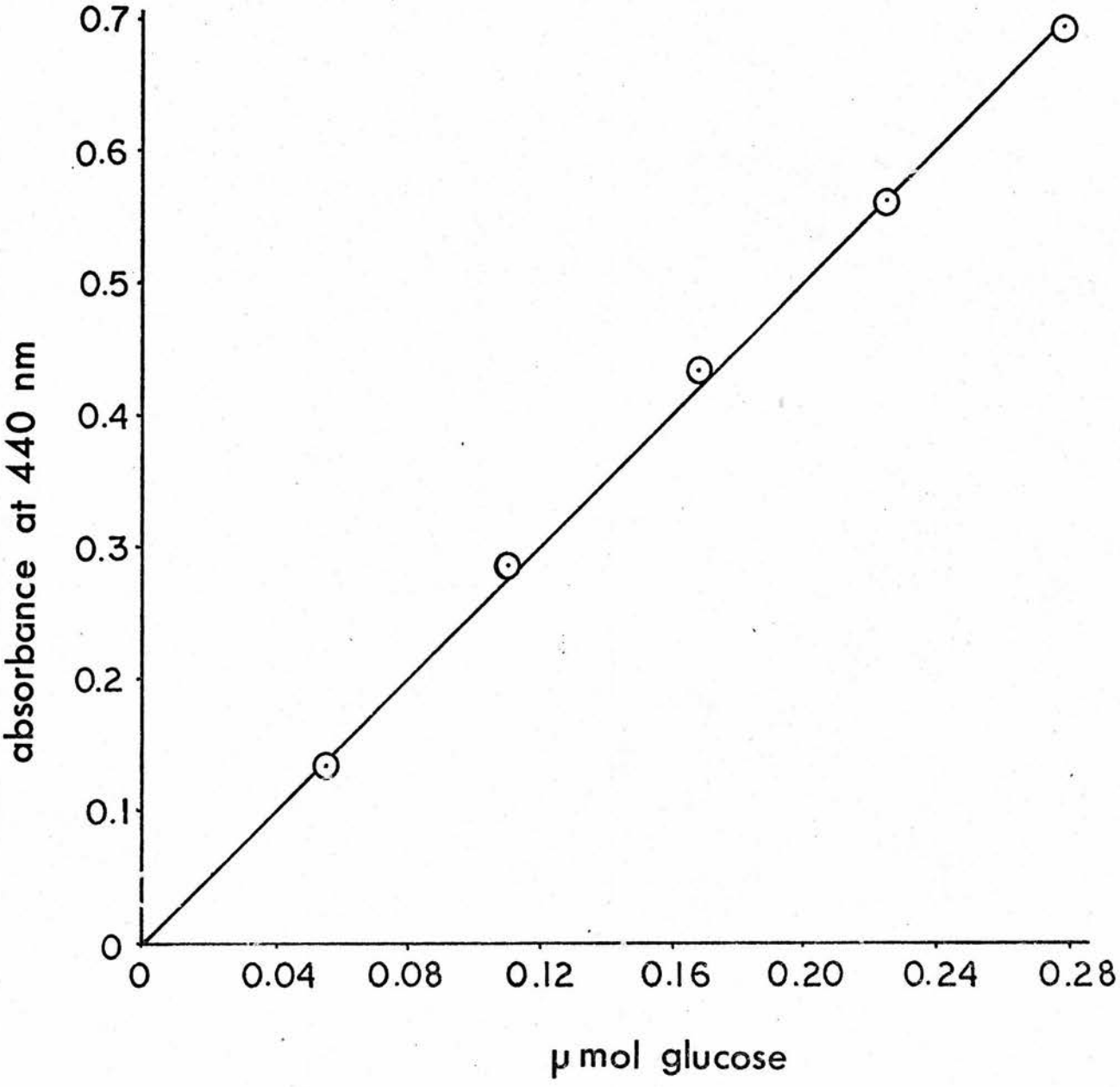


Fig. 2.1 Glucose standard curve as obtained by the method of Huggett and Nixon (1957).

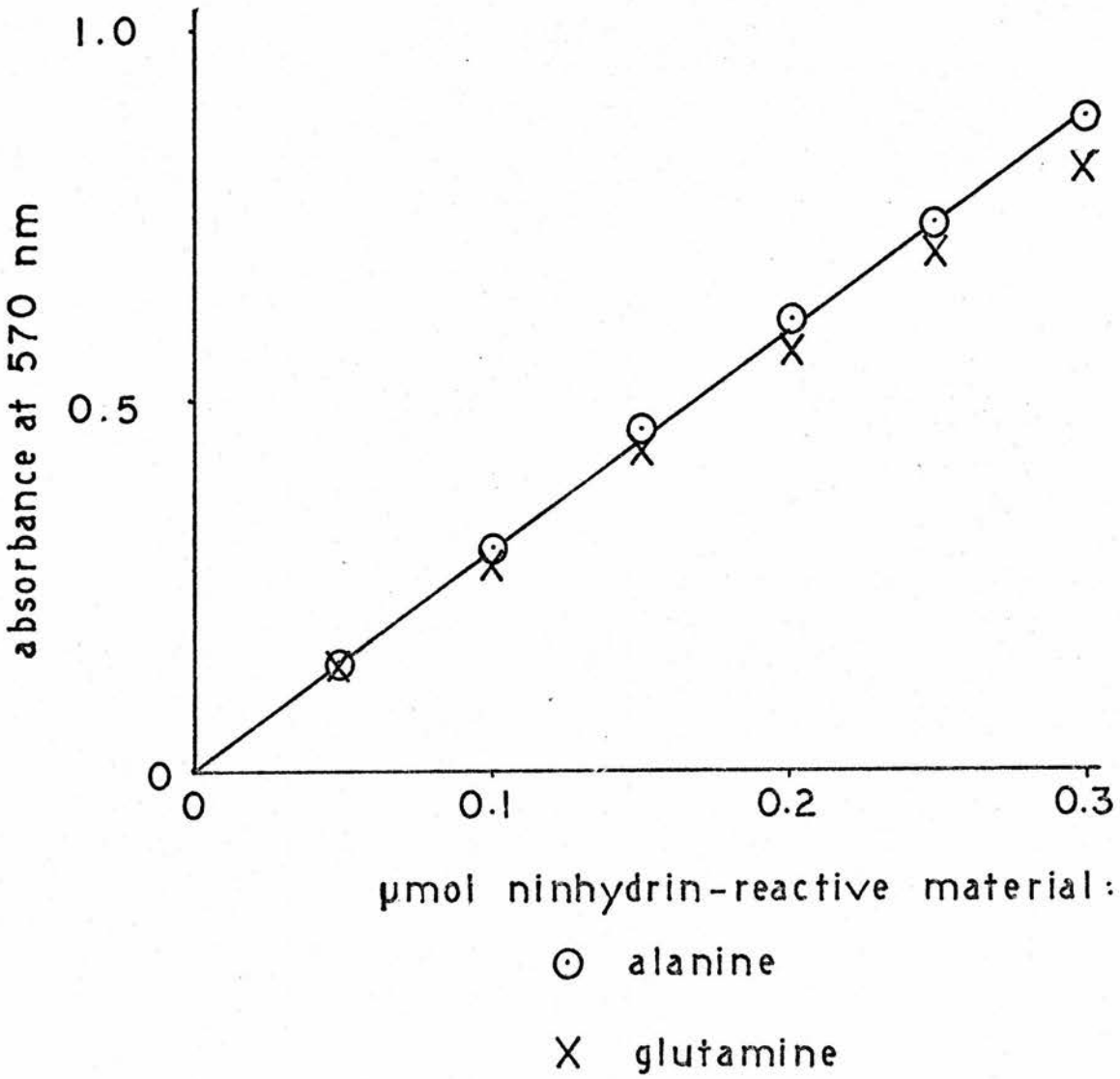


Fig. 2.2 Standard curve of total ninhydrin-reactive material by the method of Moore and Stein (1954b). Curves are shown for alanine and glutamine.

absorbance due to NADH was recorded at 340 nm.

2.2.3 Pyruvate

Pyruvate was assayed using lactate dehydrogenase and NADH in phosphate buffer at pH 7.0 (Bücher et al., 1965), recording the absorbance decrease. Pyruvate estimations were carried out on the day of perfusion, pyruvate in perchlorate extracts being determined immediately after neutralization of the extract, since pyruvate ion is unstable.

2.2.4 Total Ninhydrin-Reactive Material

The method used was that of Moore and Stein (1954b). Prior to use, the methyl-cellosolve solvent was tested for peroxides by adding it to 4% aqueous potassium iodide. In every case a pale straw colour resulted, indicative of a low peroxide content. Fresh reagent was made up for each set of determinations. Standard curves were plotted for alanine and glutamine, which did not differ greatly, and were linear from 0 to 0.3 µmoles of α-amino nitrogen per assay (Fig 2.2). Under the conditions of assay 0.1 µmoles of alanine gave an absorbance of 0.291 at 570 nm.

2.2.5 Aspartate

Transamination of aspartate with α-oxoglutarate using GOT was coupled to reduction of oxaloacetate by malate dehydrogenase and NADH (Pfleiderer et al., 1955; Pfleiderer, 1965b). This assay was straightforward.

2.2.6 Alanine

Alanine was measured in an analogous manner to aspartate, by coupling transamination to pyruvate reduction:

$$\text{Alanine} + \alpha\text{-oxoglutarate} \xrightarrow{\text{GPT}} \text{Pyruvate} + \text{glutamate}$$

$$\text{Pyruvate} + \text{NADH} + \text{H}^+ \xrightarrow{\text{LDH}} \text{Lactate} + \text{NAD}^+$$

The composition of the assay mixture was as follows:

Phosphate buffer, pH 7.2, 0.2 mol/l	0.500 ml
α-oxoglutarate, 150 m mol/l (neutralized)	0.050 ml
NADH, 8.5 mg/ml	0.020 ml
LDH, 0.1 mg/ml	0.010 ml
GPT, 10 mg/ml	0.005 ml
Distilled water to a total volume of	1.000 ml

Up to 0.4 ml of sample could be assayed; reaction was started by addition of GPT.

Two difficulties were encountered in the development of this assay. Firstly, if commercial LDH suspension (10 mg/ml) was used without dilution, the absorbance decreased rapidly prior to addition of GPT. This was traced to a lack of specificity of the LDH. which also catalyzed the reduction of α-oxoglutarate by NADH. α-oxoglutarate reduction was proportional to the LDH concentration and was not abolished by dialysis of the LDH before use. The ratio of the rate of α-oxoglutarate reduction to the rate of pyruvate reduction varied with pH or enzyme dialysis, but not with enzyme concentration:

Conditions	$\frac{\mu\text{moles } \alpha\text{-oxoglutarate/min}}{\mu\text{moles pyruvate/min}}$ (mean)
Undialyzed enzyme, pH 7.6	1.05×10^{-4}
Undialyzed enzyme, pH 7.2	2.76×10^{-4}
Dialyzed enzyme, pH 7.2	3.97×10^{-4}

Dilution of the LDH 100-fold before use abolished all appreciable interference from α-oxoglutarate reduction without increasing the time required for alanine assay.

Secondly, the reaction is slow in coming to completion owing to the high Km of GPT for alanine: the value as measured by Segal et al., (1962) is 17.5 m mol/l. It was decided not to use the kinetic assay advocated by Pfleiderer (1965a) since a large number of substances in heart extract are potentially capable of influencing the reaction rate (for example metal ions and glutathione). However, using the assay described above, reaction of a standard quantity (50 n moles) of alanine was complete in 20 min. Measurement of alanine in heart extracts required 35 min, presumably because of the presence of inhibitors (such as perchlorate ion). The rate of reaction was unaffected by the addition of pyridoxal phosphate. Economy in the use of sample was effected by measurement of aspartate and alanine in the same cuvette, by sequential addition of the appropriate enzymes.

2.2.7 The Ammonia Assay

Direct measurement of ammonia in biological materials by colorimetric methods is subject to interference from naturally-occurring amides and amines (Mondzac et al., 1965). Earlier workers freed the ammonia from interfering substances by microdiffusion from an alkaline sample to an acid recipient (Conway and Cooke, 1939a). Ammonia was measured in the recipient by titration (Reif, 1960), Nessler's reagent (Seligson and Hirahara, 1957), or by the Berthelot or phenol-hypochlorite reaction (Brown et al., 1959). The

alkaline medium required for microdiffusion could cause ammonia liberation from alkali-labile proteins and peptides (Reif, 1960), though not from glutamine in physiological concentrations (Brown et al., 1957). More recently, ammonia has been adsorbed on to an ion-exchange resin which was separated and washed before determination of the adsorbed ammonia by the Berthelot reaction (Fenton, 1962; Fenton and Williams, 1968; Kingsley and Tager, 1970). Ammonia has also been measured by ion-specific electrode (Park and Fenton, 1973).

The effects of interfering substances are likewise reduced if a more specific assay method is used, such as the reaction with α-oxoglutarate catalyzed by glutamate dehydrogenase. The decrease in the absorbance of NADH is generally measured (Kirsten et al., 1963; Mondzac et al., 1965; Müting et al., 1968). Variations in the technique include measurement of the rate of reaction in the presence of excess GluDH (Kaltwasser and Schlegel, 1966) and fluorimetric measurement of NADH disappearance (Rubin and Knott, 1967). In the present investigation, ammonia was measured by one of two methods: by direct application of the Berthelot reaction, or by the use of glutamate dehydrogenase, which is more sensitive, as well as being less subject to interference.

(a) The chemical method. This method is based on the Berthelot reaction (as described by Chaney and Marbach, 1962; Kaplan, 1965). To an aliquot of sample diluted to 1 ml were added 1 ml of phenol-nitroprusside solution (Phenol, 50 g/l; Sodium nitroprusside, 0.25 g/l) and 1 ml of alkaline hypochlorite solution (Sodium hydroxide, 25 g/l; sodium hypochlorite, 40 ml of 5% w/v solution/l). The absorbance was read at 628 nm after incubation for 5 mins at 37°C without

further dilution. Under these conditions absorbance was linearly related to concentration over the range 0 to 0.2 μ moles of ammonia, with an absorbance of 3.86 per μ mole of ammonia (Fig 2.3a). The method was not sensitive enough to measure an ammonia concentration of less than 0.02 μ moles of ammonia per assay.

(b) The enzymic method. An enzymic method based on that of Kirsten et al., (1963) was used in all determinations of ammonia in heart perfusate samples and perchlorate extracts. The reaction is shown below:

$$NH_4^+ + NADH + \alpha\text{-oxoglutarate} \xrightarrow{GluDH} H_2O + NAD^+ + Glutamate.$$

Usually sample aliquots containing 20-30 n moles of ammonia were assayed in 1 ml of test solution; reactions seldom required more than 20 min to come to completion. A standard curve using ammonium sulphate showed that reaction was complete over the range 0 to 80 n moles of ammonia per assay (Fig 2.3b). ADP was added to the reaction mixture as an activator of glutamate dehydrogenase. The assay mixture was as follows:

K_2HPO_4, 0.2 mol/l, neutralized to pH 7.0 with HCl	0.200 ml
NADH, 8.5 mg/ml	0.010 ml
α-oxoglutarate, 150 m mol/l, pH 7.0	0.030 ml
ADP, 100 m mol/l	0.010 ml
GluDH, 10 mg/ml in 50% glycerol	0.010 ml
Distilled water to a total of	1.000 ml

The reaction was started by the addition of glutamate dehydrogenase, and the absorbance decrease due to NADH disappearance was recorded at 340 nm. At least two blank reactions (without addition of sample)

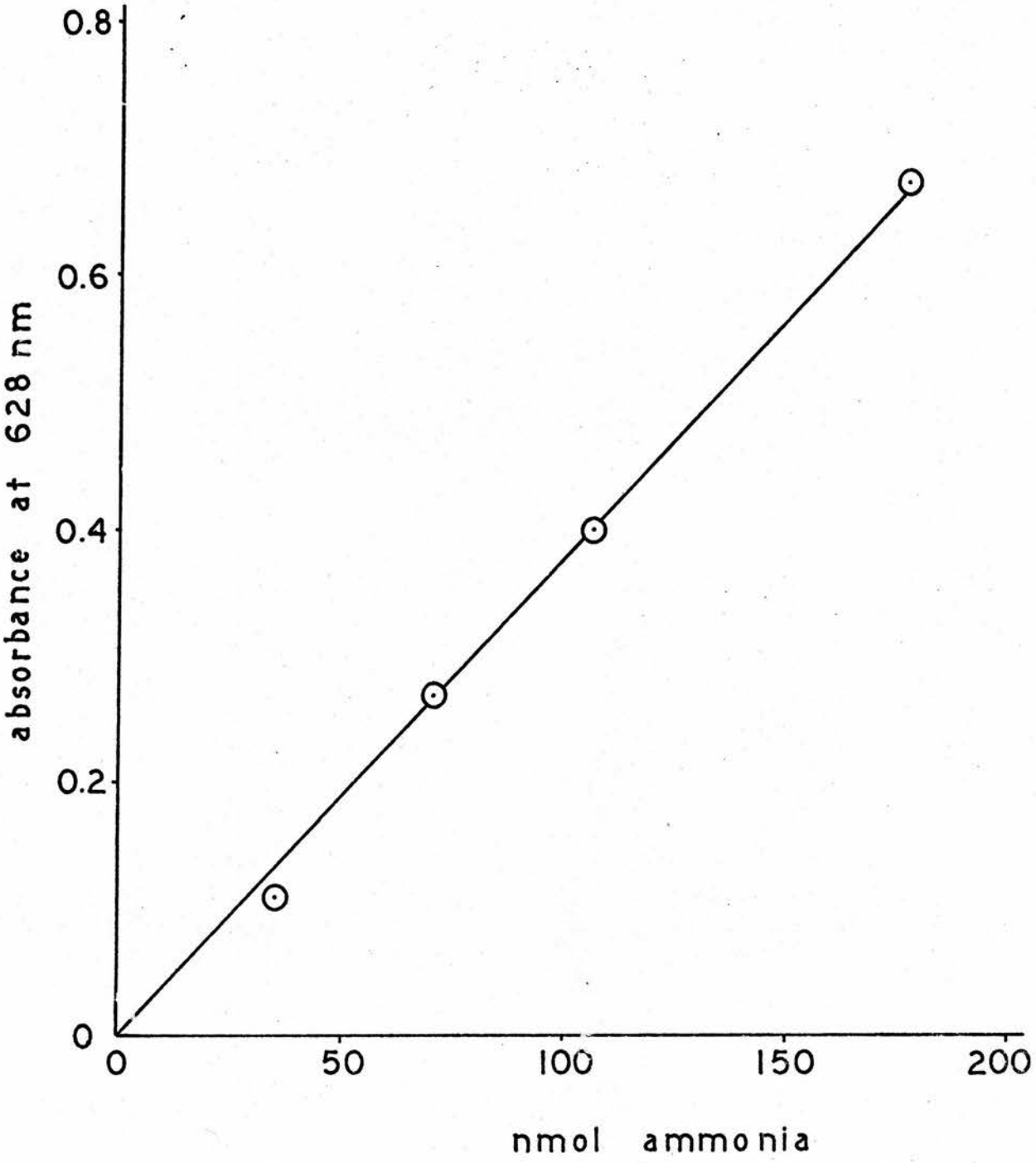


Fig. 2.3a Standard curve of ammonia assayed by the chemical method.

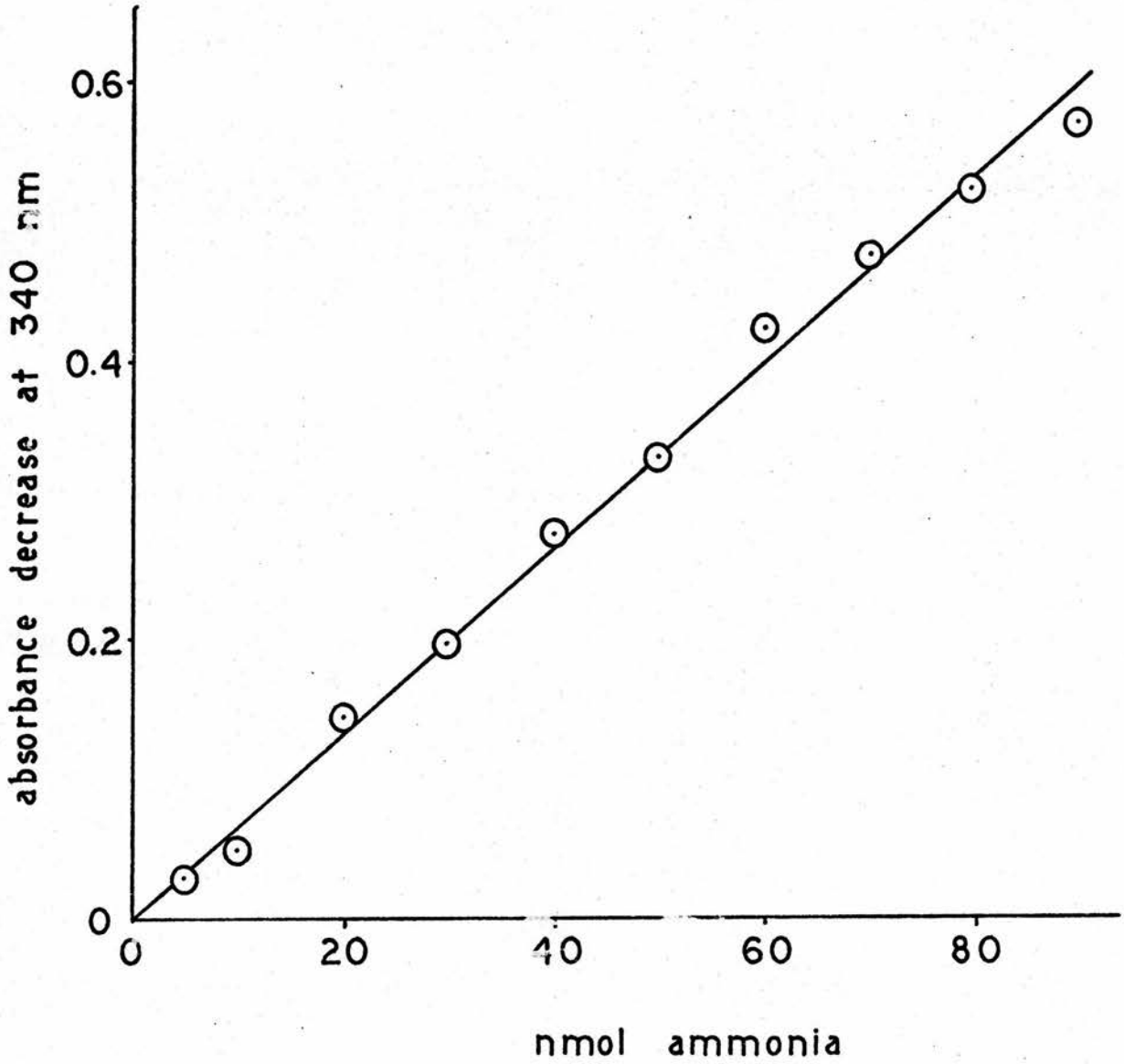


Fig. 2.3b Standard curve of ammonia assayed by the enzymic method.

Reagent	Ammonia concentration, μ mol/l	Volume of reagent used in assay, ml	Contribution of reagent to blank value in ammonia assay, as n moles of NH_4^+
NADH	0.0	0.010	0.0
ADP	0.0	0.010	0.0
α-oxoglutarate	0.0	0.030	0.0
GluDH	363	0.010	3.6
Buffer:			
(1) $NaH_2PO_4/NaOH$	55.5	(0.200)	(11.1)
(2) $KH_2PO_4/NaOH$	39.1	(0.200)	(7.8)
(3) K_2HPO_4/HCl	3.5	0.200	0.7
Total reagent blank	-	-	4.3
Krebs-Ringer	4.1	0.050 - 0.500	0.2 - 2.1
Neutralized perchloric acid	8.4	0.200	1.7

Table 2.1 The ammonia content of reagents used in the ammonia assay.

The ammonia content of each reagent was found by varying the volume of reagent added to the assay mixture. The standard mixture contained K_2HPO_4/HCl buffer; ammonia contents of buffers prepared from the dihydrogen phosphates are shown for comparison. The ammonia content of GluDH (Boehringer solution in 50% glycerol) varied between batches of enzyme. The value reported here refers to a single batch with a particularly low ammonia content which was used in all ammonia assays, without prior dialysis. The ammonia concentrations in freshly-prepared Krebs-Ringer and perchloric acid neutralized with K_2CO_3 are also shown.

were performed with every batch of estimations. Blank values reflected the presence of ammonia in reagents or adsorbed to glassware.

Since the quantities of ammonia to be measured are generally small (20-30 n moles per assay), contamination by exogenous ammonia had to be avoided. It derives from several possible sources:

1. Glassware. All glassware was washed in 0.1 mol/l HCl prior to use in ammonia experiments. Cuvettes were a common source of contaminating ammonia: if they had previously held reaction mixtures containing enzymes in ammonium sulphate suspension, they usually retained a considerable quantity of adsorbed ammonium ion. A set of cuvettes and stirrers was therefore kept specifically for ammonia estimations, and was soaked in 0.1 mol/l HCl for 30 minutes before use.

2. Distilled water. Since all reagents were prepared with distilled water, the separate contribution of this source towards the ammonia contamination is difficult to assess. It accounts for the blank absorbance decrease remaining when the contributions of all other sources have been accounted for. Glass-distilled water was used throughout the present study.

3. Laboratory air. Ammonia in laboratory air could come from rats temporarily caged in the vicinity prior to use in experiments, or from experiments producing ammonia in neighbouring laboratories. The ammonia content of laboratory air was tested by aspirating 50 litres of air overnight through 18 m mol/l sulphuric acid in a Dreschel bottle. Since this procedure caused no increase in the measured ammonia

content of the acid, it was inferred that ammonia contamination from atmospheric sources was negligible.

4. Reagents. Several of the reagents used in the ammonia assay contained ammonia which contributed to the absorbance decrease of blank tests. The individual ammonia contents found by varying the quantity of each reagent in the cuvette are shown in Table 2.1. Routine assays involved the measurement of 20-30 n moles of ammonia. The ammonia contamination introduced in the buffer was minimized (1) by using potassium rather than sodium phosphate and (2) by using dipotassium phosphate, which was adjusted to pH 7.0 with dilute HCl freshly prepared from the concentrated acid.

Loss of ammonia gas from solutions. If ammonia is lost from solution as NH_3 gas, measurements of ammonia changes during for example prolonged perfusion will be inaccurate. A trial experiment, however, showed that no such loss occurred under experimental conditions. Standard heart Krebs Ringer (250 ml, pH 7.4, 37°C) containing 4.6 m mol/l ammonia was gassed vigorously with oxygen-5% CO_2 gas mixture for 2 hours, during which time no ammonia was lost from the solution. It can therefore be assumed that the ammonia in the water phase does not enter the gas phase during closed-circuit perfusion. Furthermore, it is unlikely that any loss of ammonia occurs during overnight storage of samples at -15°C.

2.2.8 Glutamate and Glutamine

Chemical methods for assay of these compounds in biological materials are neither reliable nor specific. Although specificity can be increased by separating the compounds on an ion-exchange column

before measuring them (Bessman et al., 1948; Moore and Stein, 1954a,b), most of the techniques which have been reported depend on the use of specific enzymes.

The estimation of glutamate using glutamate dehydrogenase* is complicated by the unfavourable equilibrium constant of the reaction under physiological conditions, so that special steps are necessary in order to ensure that the reaction proceeds to completion. Sowerby and Ottaway (1966) removed the NADH formed by coupling it to tetrazolium reduction. Holzer et al., (1965) used the expensive 3-acetylpyridine analogue of NAD^+, which has a more favourable redox potential than NAD^+, but reacts more slowly and requires a higher enzyme concentration. Raising the pH alters the equilibrium so as to increase glutamate oxidation; this is enhanced by the use of a ketone reagent such as hydroxylamine (Albers et al., 1961) or hydrazine (Bernt and Bergmeyer, 1965) to remove the α-oxoglutarate formed in the reaction.

Glutamine cyclizes to ammonium pyrrolidone-carboxylate when heated in solution at neutral pH. Hence the decrease in ninhydrin-liberated CO_2 after this treatment, measured manometrically, has been used as a measure of glutamine concentration (Hamilton, 1945). Glutamine has also been determined directly using glutamine amino-transferase (Kupchik and Knox, 1970). It is more usual to measure glutamine by first hydrolysing it to glutamate and ammonia.† Acid hydrolysis is simple (Segal and Wyngaarden, 1955; Balis, 1971), though

* Glutamate + NAD^+ and $H_2O \xrightarrow{\text{GluDH}}$ α-oxoglutarate + NADH + NH_4^+

† Glutamine + $H_2O \longrightarrow$ Glutamate + NH_3

enzymic hydrolysis is more specific. Asparaginase has been used in

a two-step procedure (Cooney et al., 1971): a small quantity of the enzyme is used to hydrolyse asparagine, after which a large amount is added to hydrolyse glutamine. The procedure is rather expensive. Glutaminase, however, is the enzyme of choice, and has been used in glutamine determination for some years (Archibald, 1944a,b; Segal and Wyngaarden, 1955; Sowerby and Ottaway, 1966; Ottaway, 1969a; Nahorski, 1971; Williams et al., 1972). Commercial enzyme preparations are unstable but a less pure laboratory preparation is more stable and perfectly satisfactory (Sowerby and Ottaway, 1966). Hydrolysis of glutamine is followed by measurement of ammonia (Archibald, 1944a; Segal and Wyngaarden, 1955) or glutamate (Sowerby and Ottaway, 1966; Ottaway, 1969a; Nahorski, 1971; Williams et al., 1972). Older methods for the estimation of glutamate and glutamine were reviewed by Archibald (1945); more modern methods have been reviewed by Balis (1971).

2.2.3.1 Laboratory Procedure - Glutamate

(a) The tetrazolium method (Sowerby and Ottaway, 1966). Glutamate oxidation was coupled to the reduction of INT using phenazine methosulphate. The formazan produced was dissolved by adding acetone and dilute hydrochloric acid and its absorbance was measured at 460 nm. Absorbance was linearly related to concentration from 0 to 0.25 μ moles of glutamate, giving an extinction coefficient of 2.12 per μ mole.

The method had several disadvantages. Firstly, it was not sufficiently sensitive to detect the low glutamate concentrations in a rat heart perfusate. Secondly, variation between batches of

Assay substrate		Volume of 1 mol/l nitron acetate added, ml	Precipitate of INT-perchlorate on adding INT	Glutamate measured as % of control value
0.1 µmol glutamate +	½ ml H_2O (control)	0.0	No	100
	½ ml neutralized $HClO_4$	0.0	Yes	77
	"	0.005	Yes	81
	"	0.010	Slight	102
	"	0.020	No	75
	"	0.050	No	22
	½ ml H_2O	0.020	No	74

Table 2.2 Inhibition of glutamate assay by excess perchlorate or excess nitron.

To an aliquot of standard containing 0.1 µmol of glutamate was added 0.5 ml of H_2O or 0.5 ml of perchloric acid 1 mol/l, neutralized with K_2CO_3. After addition of 1 mol/l nitron acetate and mixing, the mixture was centrifuged and glutamate was assayed in the supernatant by the method of Sowerby and Ottaway (1966). The presence or absence of a precipitate on addition of INT was recorded, and the recovery of glutamate was calculated as a percentage of the value measured in the absence of perchlorate and nitron.

Concentration in assay mixture		Percentage oxidation of standard glutamate in assay
Hydroxylamine, mol/l	Ammonia, mmol/l	
0.03	0.0	56.3
0.06	0.0	78.2
0.15	0.0	100.0
0.28	0.0	100.0
0.15	0.0	100.0
0.15	0.36	92.4
0.15	3.6	104.0
0.15	18.0	99.8

Table 2.3 The effect of hydroxylamine and ammonia concentration on the equilibrium of the glutamate dehydrogenase reaction used in glutamate assay.

A standard aliquot of glutamate (0.050 µmoles) was assayed using the hydroxylamine technique (see text). The hydroxylamine concentration in the assay mixture was varied, or ammonium sulphate solution was added to the cuvette, to produce the conditions shown. 100% oxidation of glutamate occurred when 0.050 µmoles of NADH were generated in the reaction, as measured by absorbance at 340 nm.

INT resulted in different absorbance coefficients (see Sowerby and Ottaway, 1966). Thirdly, the method was unsuitable for assay of perchlorate acid extracts. The small amount of perchlorate remaining after neutralization formed an insoluble salt with the tetrazolium cation, reduction of which was impaired. Perchlorate can be removed by the addition of nitron acetate, since nitron forms an insoluble perchlorate which can be centrifuged down before addition of INT. Excess nitron itself impaired tetrazolium reduction (Table 2.2), and the difficulty of achieving exact stoicheiometry of nitron addition rendered the technique impracticable.

(b) High pH and Ketone Reagent

1. Hydroxylamine (Albers et al., 1961). The assay mixture was as follows:

Hydroxylamine, 0.3 mol/l, pH 8.0	0.5 ml
NAD^+, 100 m mol/l	0.020 ml
Glutamate dehydrogenase (10 mg/ml solution in 50% glycerol)	0.010 ml
Distilled H_2O to a total of	1.000 ml

The increase in NADH absorbance was complete in 20 minutes at 37°C, corresponding to complete oxidation of the glutamate added. The rate of reaction was not increased by the addition of ADP, an activator of glutamate dehydrogenase in the reverse reaction. The percentage of glutamate oxidized was affected by the hydroxylamine concentration in the cuvette, but not by the addition of ammonia (Table 2.3). It was therefore concluded that the generation of ammonia during the reaction would have no detrimental effect on the measurement of glutamate.

The hydroxylamine method was a simple and reliable technique, used when glutamate was to be assayed without concomitant measurement of glutamine.

2. Hydrazine (Bernt and Bergmeyer, 1965). The buffer used in this method contained glycine (0.9 mol/l) and hydrazine (0.72 mol/l) at pH 9.0. The rate of reaction was rather slower than that in the hydroxylamine technique, but hydrazine was the only method which could be used when glutamine was also to be assayed (see below).

Some assays were affected by a sharp increase in absorbance beginning about 10 minutes after enzyme addition, which was unconnected with NADH production. It occurred in cuvettes containing only glutamate dehydrogenase and buffer, and the solution then exhibited a "pearly" turbidity. It was concluded that enzyme was denatured and precipitated by the hydrazine or by the high pH. The effect was more pronounced at lower buffer concentrations, suggesting that glycine might have a protective effect on the enzyme. It did not occur if glutaminase preparation had been added, suggesting that the protein in this preparation might likewise protect the enzyme. Consequent experiments showed that defatted bovine serum albumin completely abolished the spurious rise in absorbance, and this was included in all assay mixtures (5 mg per ml of reaction mixture). The composition of the assay mixture was as follows:

Glycine/Hydrazine buffer (see above), pH 9.0	0.500 ml
NAD^+, 100 m mol/l	0.020 ml
Bovine serum albumin (10% in water)	0.050 ml
GluDH, 10 mg/ml in 50% glycerol	0.010 ml
Distilled water to a total of	1.000 ml

2.2.8.2 Glutamine

(a) Acid hydrolysis (Adapted from Balis, 1971). Aliquots of sample, containing 20 to 100 n moles of glutamine, were pipetted into 1 ml Eppendorf microbeakers and heated in 2 mol/l HCl for 15 minutes at 100°C. Ammonia was measured in the hydrolysate after neutralization with a calculated amount of NaOH. The glutamine content was found by subtracting the ammonia concentration measured in the unhydrolysed sample from that measured in the hydrolysate. If ammonia greatly exceeded glutamine in the sample, as it did in perfusate to which ammonia had been added, it was more accurate to measure glutamate in the hydrolysate, using the hydroxylamine method (2.2.8.1). Acid hydrolysis was used in order to avoid the lengthy preparation of glutaminase when only a small number of samples required to be analysed.

(b) Enzymic hydrolysis. Hydrolysis of glutamine by glutaminase, being more specific than acid hydrolysis, was used whenever practicable. The method of Sowerby and Ottaway (1966) based on that of Klingman and Handler (1958) yields a glutaminase preparation which is stable on storage and is effective in the measurement of glutamine. A specimen preparation is appended (Appendix 2.I). It was possible to couple glutamine hydrolysis directly to oxidation of the glutamate formed, recording the absorbance increase due to NADH produced in the GluDH reaction.

Glutaminase was inhibited by both ketone reagents used in glutamate measurement, and also by the perchlorate ion present in tissue extracts. Inhibition in all cases increased with the time for which the enzyme was in contact with the inhibitor. These

Time of preincubation of glutaminase in buffer before addition of glutamine, min	Relative rate of glutamine hydrolysis in given buffer			
	Hydroxylamine pH 8.0	Glycine/Hydrazine pH 9.0	Glycine/Hydrazine, pH 9.0	
			+0.1 ml neutralized perchloric acid	+0.4 ml neutralized perchloric acid
0	92	81	80	57
4	18	56	47	15
10	6.1	63	38	16
20	6.0	53	26	12

Table 2.4 The inhibition of glutaminase by hydroxylamine, hydrazine and perchlorate ion.

The initial rate of glutamine hydrolysis was measured in the given buffer in a system containing 2 mmol/l NAD, 100 mmol/l potassium phosphate, 200 µg GluDH and 5 µl glutaminase preparation, in a total volume of 1 ml, at 37°C. Buffers were 0.15 mol/l hydroxylamine, pH 8.0; or 0.36 mol/l hydrazine + 0.45 mol/l glycine, pH 9.0; to the latter were added in certain experiments 0.1 ml or 0.4 ml of perchloric acid, 1 mol/l, neutralized with K_2CO_3. Reaction was started by the addition of 0.2 mol/l glutamine, with or without preincubation of glutaminase in the buffer system for the time stated. The rate of glutamine hydrolysis measured in phosphate buffer at pH 7.0 by the method described in Appendix 2.I is taken as 100.

Fig. 2.4 Inhibition of glutaminase by substances present in the assay mixture for glutamine.

The graph shows the decrease in the initial rate of glutamine hydrolysis as the time increases for which glutaminase is incubated with the inhibitor at 37 °C. The units of the relative reaction rate, and the experimental method, are explained in the legend to table 2.4.

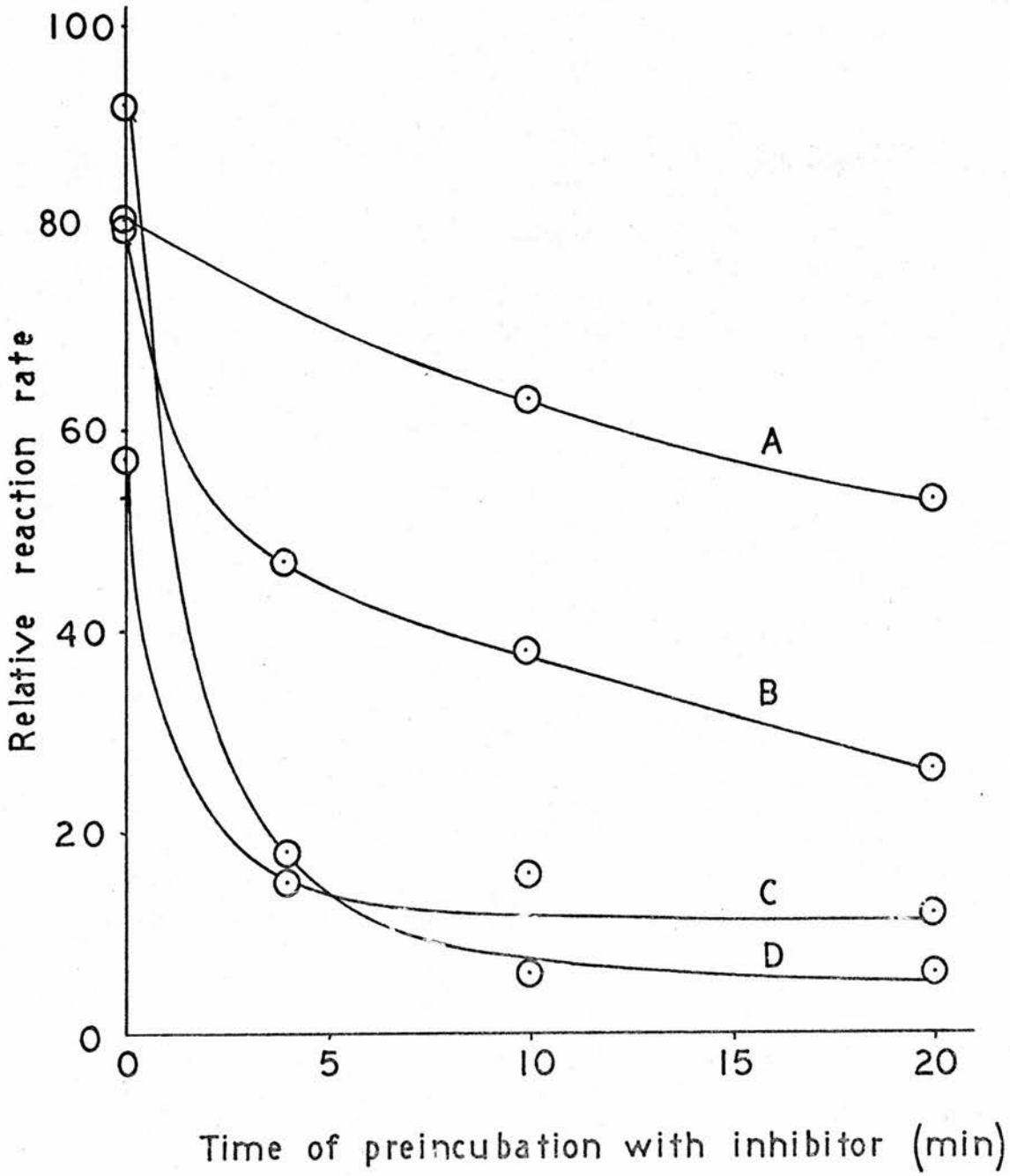
The nature of the inhibitor in the reaction medium is as follows:

A = glycine-hydrazine buffer, pH 9.0.

B = glycine-hydrazine buffer + 0.1 ml neutralized $HClO_4$, 1 mol/l.

C = glycine-hydrazine buffer + 0.4 ml neutralized $HClO_4$, 1 mol/l.

D = hydroxylamine, pH 8.0.


100
80
60
40
20
0
Relative reaction rate
0
5
10
15
20
Time of preincubation with inhibitor (min)
A
B
C
D

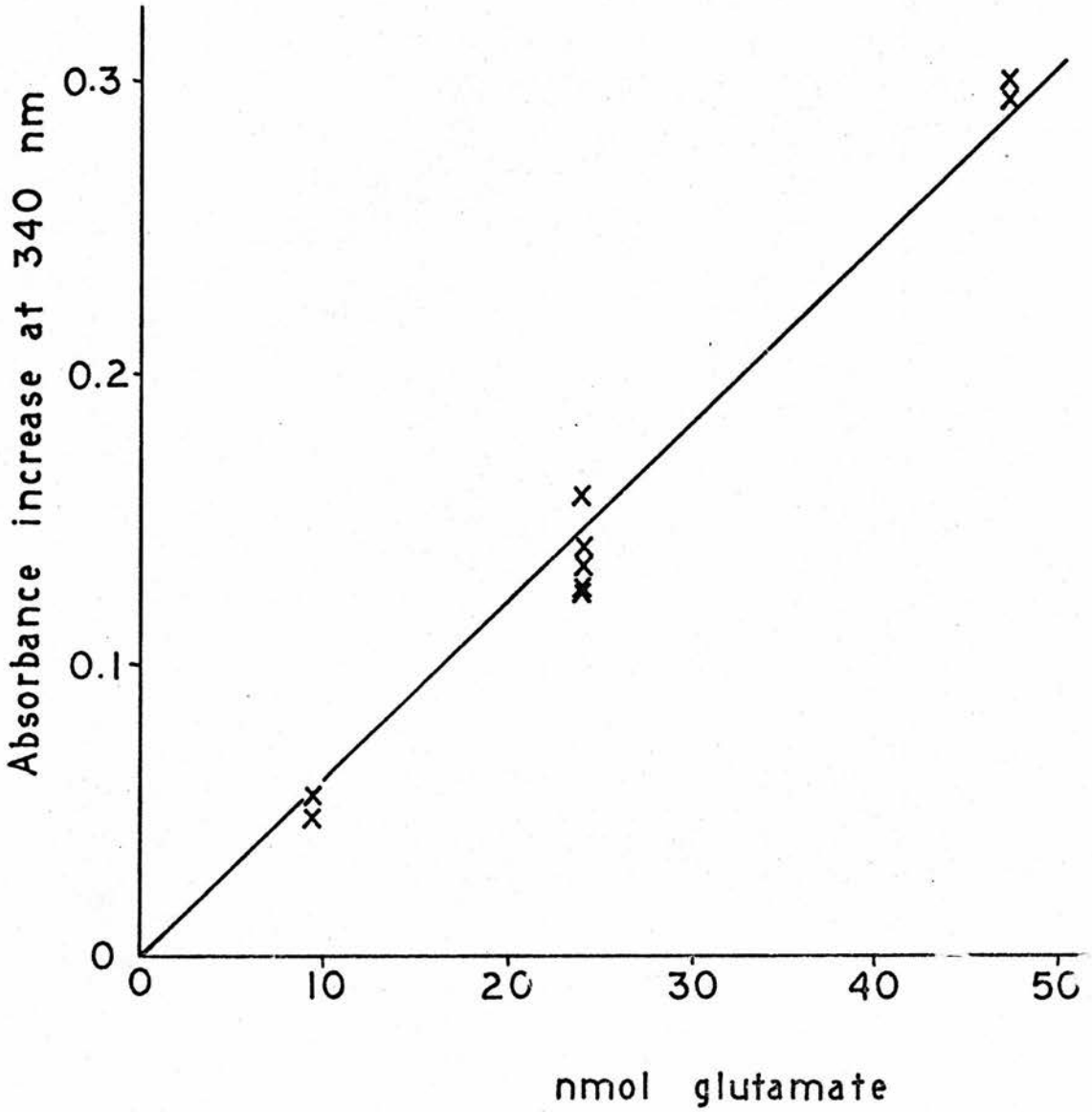


Fig. 2.5a Standard curve of glutamate measured by the use of glutamate dehydrogenase in glycine-hydrazine buffer.

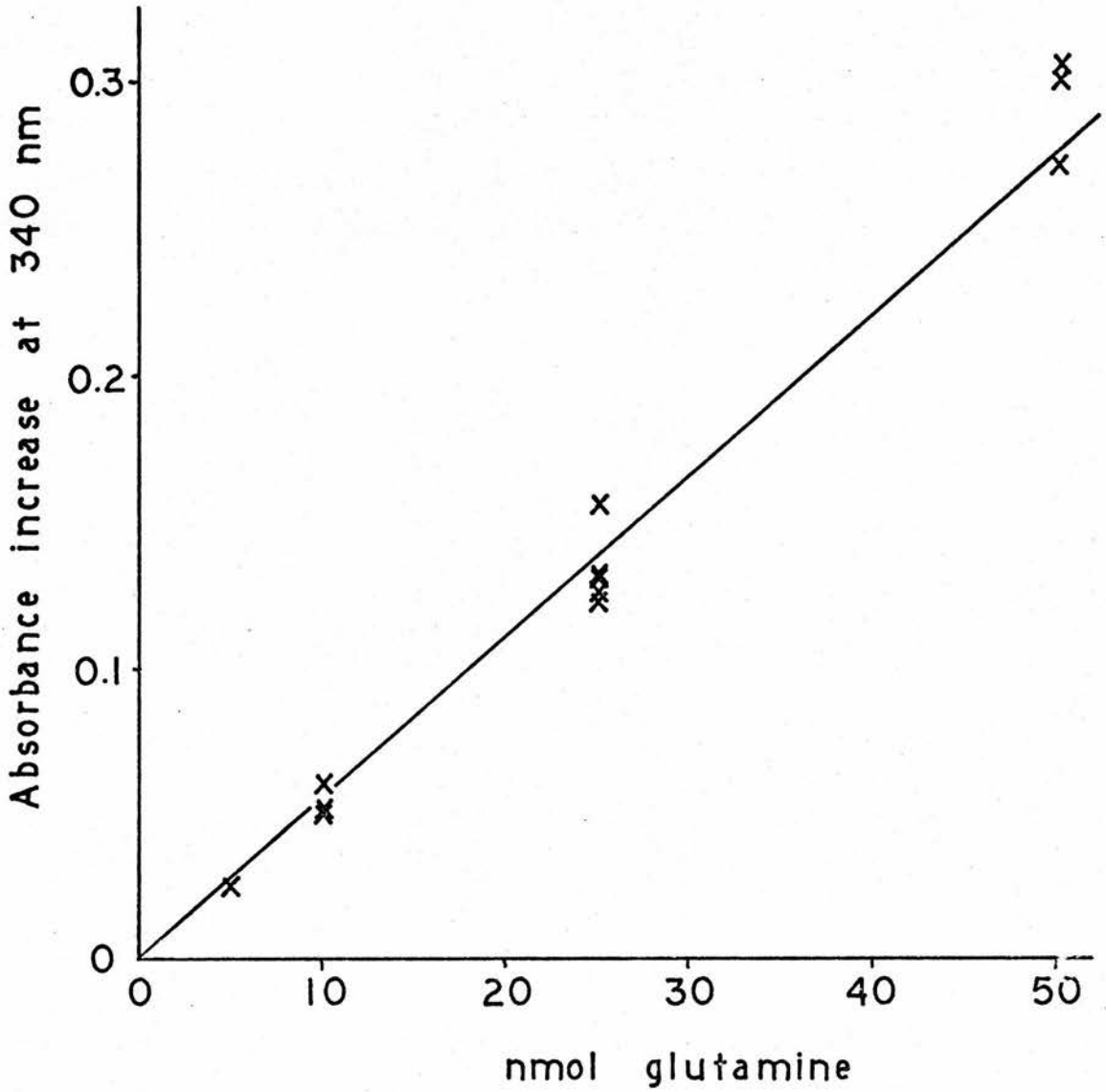


Fig. 2.5b Standard curve of glutamine measured using glutaminase in glycine-hydrazine buffer after prior determination of glutamate.

effects are shown in Table 2.4 and Fig 2.4. Glycine-hydrazine buffer was used in routine glutamine assay in preference to hydroxylamine, and the amount of perchlorate added was kept to a minimum: 50 µl of extract was sufficient for glutamine determination. Phosphate was added as an activator of glutaminase (Klingman and Handler, 1958; Sowerby and Ottaway, 1966).

2.2.8.3 The determination of glutamate and glutamine in the same cuvette

Glutamate and glutamine were determined in the same cuvette, using the following assay mixture:

Buffer: Glycine 0.9 mol/l, hydrazine 0.72 mol/l, pH 9.0	0.500 ml
Potassium phosphate, 1 mol/l, pH 9 (approx)	0.050 ml
Bovine serum albumin, 10% in H_2O	0.050 ml
NAD^+, 100 m mol/l	0.020 ml
Sample aliquot	
GluDH, 10 mg/ml in 50% glycerol	0.020 ml
Glutaminase in 50% glycerol (Appendix I)	0.050 ml
Distilled water to a total of	1.000 ml

All the adducts except the two enzymes were mixed in the test cuvette. A blank from which sample was omitted was placed in the reference beam of the spectrophotometer. The addition of glutamate dehydrogenase to sample and reference cuvettes caused a rise in absorbance equivalent to the glutamate content of the mixture, complete in 15-20 minutes; on addition of glutaminase, the further rise indicated glutamine and was complete in a further 35 minutes.

Standard curves were drawn for glutamate and glutamine. The absorbance increase in 1 ml of solution due to 1 µ mole of glutamate

using this technique was 6.08 and that due to 1 μmole of glutamine was 5.52 (Fig 2.5).

2.2.9 Total Amino-acid Analysis

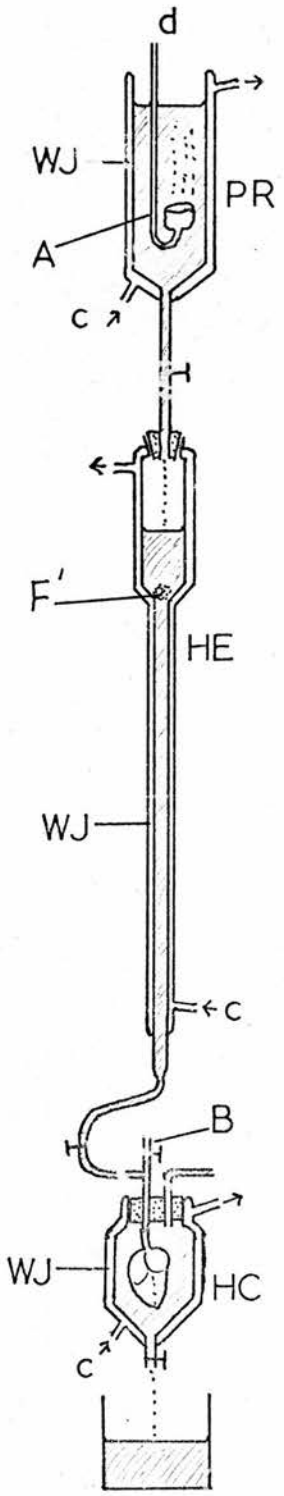
In a small number of hearts, amino acids in tissue and perfusate were measured using a Locarte amino acid analyzer (see Moore and Stein, 1954a,b). By this method a large number of amino acids can be measured in a single sample, although glutamine elutes with threonine and cannot be distinguished from it. Ammonia cannot be measured accurately by this method: the heavy contamination of the reagents by ammonia always gives a large peak. Samples for analysis must contain no amino acid in excess of 0.6 μmoles; and a pH of 2.2 is necessary to ensure that all amino acids adhere to the resin. Samples were prepared as follows:

An appropriate aliquot of solution (3-5 ml of perfusate samples, or 0.2 ml of tissue extracts) was acidified to pH 2 (Johnson Universal Indicator Paper) with hydrochloric acid, lyophilized and stored at -15°C. When required for analysis, the dry powder was dissolved completely in 0.5 ml of citrate buffer, 0.2 mol/l, pH 2.2. A measured aliquot (0.36 - 0.41 ml) was applied to the column and eluted with successive changes of buffer in a standard procedure. Eluted amino acids were reacted automatically with ninhydrin and recorded as peaks on a chart recorder. The area under each peak was measured and the amount of each amino acid present was calculated with reference to known standards. Glutamine, taurine and glutathione standards were run independently in order to determine their elution time and absorbance in the ninhydrin reaction.

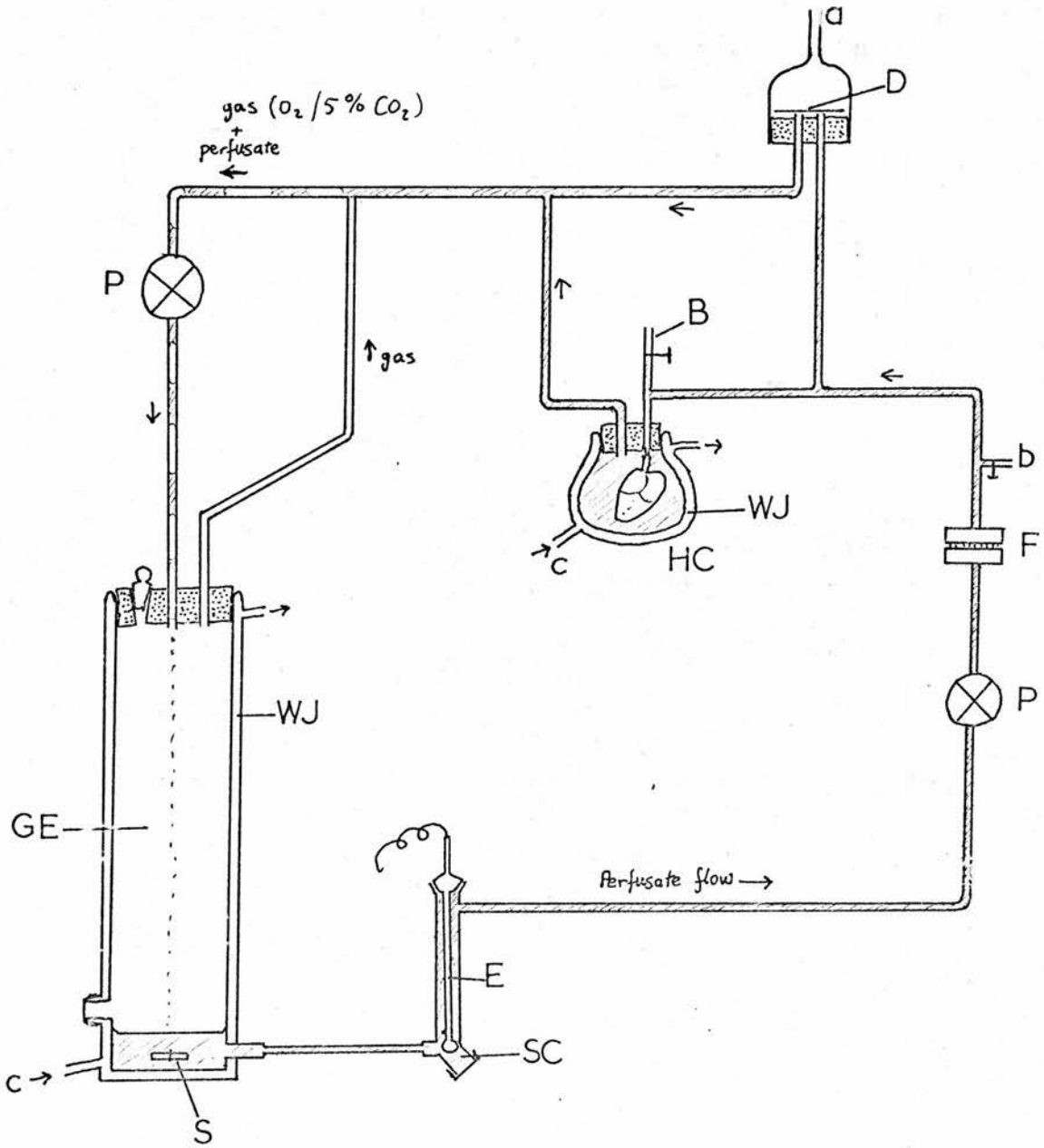
Figure 2.6

The apparatus used in perfusion of rat heart. Items are labelled as follows:

PR	Preperfusion reservoir
A	Aerator
HE	Heat exchanger
HC	Heart chamber
WJ	Water jacket
GE	Perfusate chamber and gas exchanger
S	Stirrer magnet
P	Pump
F	Sintered glass filter, porosity 3, diameter 20mm
D	Rubber diaphragm in by-pass valve
B	"Bubble-trap"
SC	Sample chamber
E	pH electrode
a	from air pressurizer
b	to manometer
c	from 37°C water bath
d	from gas cylinder (O_2/5%CO_2)
F′	Filter



Preperfusion System



Perfusion system

2.3 Miscellaneous Techniques.

2.3.1 Rats

The animals used in the present study were female albino rats of a local strain. They were maintained on a standard rat-cake diet (Oxoid 41B) with free access to water. Food, but not water, was withdrawn 18 hours prior to killing. The animals used weighed 150 - 200 g.

2.3.2 Perfusion Technique

2.3.2.1 The perfusion apparatus. The apparatus was based on that of O'Brien (1969) and was exactly as described in detail by Mowbray (1969), with two additions: the perfusion pressure could be measured directly by a manometer connected at the level of the cannula tip; and the sample chamber was modified in some experiments so as to accommodate a pH electrode (dual type micro-electrode, E.I.L., England). The apparatus is shown diagrammatically in Fig 2.6.

2.3.2.2 The perfusion medium. The medium was based on that of Krebs and Henseleit (1932). The concentrations of calcium chloride and magnesium sulphate were reduced by half, since in the live rat about half the circulating calcium and magnesium is protein-bound and thus not directly available to the tissue (see Fisher and O'Brien, 1972; Mowbray, 1969). Calcium in stock solutions was measured by the oxalate titration method of Kramer and Tisdall (1921).

The pH of the medium thus constituted from Analar reagents was 7.57. The difference in pH from that quoted by Krebs and Henseleit (1932) was partly due to the reduction in the concentration of the acidic calcium and magnesium salts. Replacing one-third

of the bicarbonate with sodium chloride yielded a medium of pH 7.39. Bicarbonate was well gassed with oxygen-5% CO_2 (British Oxygen Company) before the addition of the other components in order to avoid the precipitation of calcium and magnesium salts (Krebs and Henseleit, 1932) due to high pH. When medium of pH 7.57 was required, the original bicarbonate concentration of Krebs and Henseleit was used. Some experiments required a medium in which the standard potassium concentration was reduced to 60%, achieved by replacing half the potassium chloride with sodium chloride. All perfusion media contained 1 mg per ml glucose (5.56 m mol/l).

About 200 ml of the medium was poured into the reservoir of the preperfusion system, and allowed to run to the bottom of the system without gathering air-bubbles. 50 ml was poured into the perfusate chamber, and both portions of medium were gassed continuously. The cannula was attached to the bottom of the preperfusion system and filled to the tip with fluid, again excluding air-bubbles. The pump was started, circulating the medium in the closed system. When the temperature of the medium had reached 37°C and its pH had become steady, it was ready for the attachment of the heart.

2.3.2.3 Preparation of the heart. Rats were anaesthetized in an ether-air mixture. The beating heart was removed as quickly as possible, avoiding injury to it, leaving the aortic arch intact. It was placed in ice-cooled saline (0.9%, containing 0.1 m mol/l EDTA to reduce blood clotting) until it had become quite cold. It was then cleaned, and all adhering tissue was removed. The aorta was cut just on the proximal side of the first branch. The heart was blotted and transferred to a tared beaker containing about 30 ml of ice-cold

Krebs medium and weighed. The end of the aorta was then slipped over the tip of the cannula, connected to the preperfusion system, ensuring that no air-bubbles entered during the process. The aorta was tied on to the cannula with silk thread, and the lower tap on the preperfusion system was opened to allow medium to flow through the heart. Heart beat was often irregular at first. Hearts which were not beating satisfactorily after 5 minutes or which showed patches of myocardium from which blood had not cleared, were rejected.

2.3.2.4 Perfusion and sampling. The heart was washed out in the preperfusion system for ten minutes. It was then transferred as rapidly as possible to the closed system without allowing any air-bubbles to pass through the organ. In general hearts were perfused for a period of 75 minutes, although if required, a viable preparation could be maintained for up to 2 hours.

Samples were taken by hypodermic syringe from a chamber (Fig 2.6), fitted with a "suba-seal" rubber cap. In most experiments 1 ml of perfusate was withdrawn every 15 minutes. Samples were heated to 90°C for 5 minutes to inactivate enzymes released by the heart; if this was not done the presence of glutamate dehydrogenase interfered with the ammonia assay, and LDH interfered with pyruvate measurement. No loss of added glutamine (500 μ mol/l) due to this step was seen. Samples were stored at -15°C.

The values of some physical and metabolic parameters in rat hearts perfused under the conditions described are shown in Table 2.5. The pH of the perfusing fluid remained almost constant throughout perfusion, falling during 75 minutes by 0.01-0.02 pH units.

Heart rate	170-200 beats/min
Oxygen consumption (control perfusion)	3.6 µmol/min/heart
" " (+ 300 µmol/l NH_4^+)	4.5 "
Tissue lactate/pyruvate ratio (6 hearts)	12.1 ± 4.5 (mean ± S.E.M.)
Perfusate lactate/pyruvate ratio (7 hearts)	13.3 ± 7.6
Glucose uptake (10 hearts)	32.5 ± 13.7 µmol/g/hour
Lactate production (11 hearts)	24.4 ± 10.0 "
Temperature (in heart chamber)	37°C
pH of perfusate	7.39 or 7.57
Perfusion pressure (at cannula tip)	70 cm of H_2O

Table 2.5 Some parameters of the perfused rat heart.

2.3.2.5 Preparation of tissue extracts. At the end of perfusion, the heart was removed from the heart chamber and rapidly crushed in a Wollenberger clamp pre-cooled in liquid nitrogen. Perfusate flow was maintained until the heart was frozen. The frozen tissue was transferred without delay to 1 ml of 1 mol/l perchloric acid in 20% v/v ethanol, pre-cooled in an ice-salt mixture, homogenized (Ultraturrax Homogenizer, Janke and Kunkel) and centrifuged. The soluble extract obtained was neutralized with potassium carbonate and cooled to freezing point, and the potassium perchlorate precipitated was spun down. Extracts were stored in glass vials at -15°C. If storage of unhomogenized tissue was unavoidable, it was stored immersed in liquid nitrogen. Control hearts, described hereafter as "unperfused", were analyzed after 10 min preperfusion.

2.3.3 Perfusate Contamination of Tissue Extracts

When a perfused heart is freeze-clamped in the manner described in the foregoing section, the extracellular fluid and any perfusate circulating through the heart are frozen in addition to the intracellular fluid. These cannot be entirely excluded from the tissue extract. If a metabolite to be determined in the ICF is also present in the ECF and perfusate, then the total volume of this extracellular material must be known in order that an appropriate correction may be applied. The phrase "perfusate contamination" is used here to denote this volume. It was measured by one of the procedures described below.

(a) Chloride estimation. The titrametric method of White (1961), applied to perfused rat heart by Mowbray (1969), was employed. A correction was required since chloride is also present

in the intracellular fluid, where its concentration is assumed to be 25% of that in the perfusate (Mowbray, op. cit.). However, this concentration ratio may be affected by altering the concentration of other ions in the perfusion medium, for example, by adding ammonium ion (see Lux, 1971). In the present study, two hearts perfused with medium containing 360 μ mol/l ammonia showed high internal chloride concentrations as measured by this method. Furthermore, unexplained low titres were given repeatably by certain perfusate samples, and it was considered that more accurate results could be obtained by the use of a labelled extracellular marker.

(b) ^{14}C-sorbitol as an extracellular marker (Morgan et al., 1961). Sorbitol is confined to the extracellular space. 1 μC of U-^{14}C-sorbitol and 25 mg of unlabelled sorbitol carrier were added to the 50 ml of perfusion fluid. At the end of the perfusion, radioactivity was measured by scintillation counting, both in the perfusate and in the perchlorate extract (Packard Tri-Carb liquid scintillation counter). Hence the perfusate contamination of the extract was simply and directly determined.

2.3.4 Extracellular pH

Extracellular pH was measured by incorporating a "dual" pH electrode, specially made for measuring pH in small volumes, in the main perfusion system. The electrode was connected to a pH meter: both meter and electrode were products of E.I.L. Instruments, England. The meter output was fed into a Servoscribe recorder, and adjusted so that a pH change of 1 unit (7.0 to 8.0) gave a full scale deflection (20 cm) on the recorder. A 500 μF capacitor was

connected across the recorder terminals to reduce noise caused by the operation of the perfusate pump. The recorder chart was set in motion at 12 cm per hour, and the external pH was recorded throughout the perfusion.

2.3.5 Intracellular pH

Intracellular pH was investigated by the method of Waddell and Butler (1959). 5,5-dimethyl-2,4-oxazolidinedione (DMO) is a weak acid; in solution it exists in undissociated (HDMO) and dissociated (DMO^-) forms, which are in equilibrium. The proportion of HDMO is increased by decreasing the pH:

$$H^+ + DMO^- \rightleftharpoons HDMO$$

It is assumed that the cell membrane is permeable to HDMO but not to DMO^-, and that the rate of diffusion of HDMO across the membrane is proportional to its concentration. Thus the ratio of DMO concentration between the two sides of the membrane depends on the pH gradient across it, and the internal pH can be calculated if the external pH and the internal and external DMO concentrations are measured. They are related by the equation

$$pH_i = pK + \log_{10} \left\{ \left[{}^{Ct}/_{Ce} \left(1 - \frac{Ve}{Vi} \right) - \frac{Ve}{Vi} \right] \left[10^{(pH_e - pK)} + 1 \right] - 1 \right\}$$

(Waddell and Butler, 1959), in which pHi and pHe are the internal and external pH, pK is the acid dissociation constant of DMO (= 6.13), Ce and Ct are the DMO concentrations in the ECF and total tissue water respectively, and Ve and Vi are the volumes of the extracellular and intracellular compartments. If the concentration

of DMO in the ICF can be determined directly, as it can in the perfused rat heart by application of the "perfusate contamination" correction (Section 2.3.3), the equation reduces to the following:

$$pH_i = pK + \log_{10} \left\{ {}^{C_i}/_{C_e} \left[10^{(pHe - pK)} + 1 \right] - 1 \right\}$$

$$\text{or } pH_i = pK + \log_{10} \left\{ \frac{T}{P} \cdot \frac{1}{V_i} \left[10^{(pHe - pK)} + 1 \right] - 1 \right\}$$

where Ci is the DMO concentration in the ICF, Vi is the intracellular volume per g tissue, T is the weight of DMO per g tissue ($T = V_i \times C_i$), and P is the DMO concentration in the blood or perfusate. It is assumed that P and Ce are equal. The internal pH of perfused rat heart was calculated from measurements of perfusate pH, and of DMO concentration in the perfusate (P) and in the tissue (T).

Laboratory Procedure

DMO was added to perfusion media before the start of perfusion (12.5 mg per 50 ml of medium). After perfusion its concentration was determined both in the perfusate and in the tissue, as follows: 0.5 ml aliquots of perchlorate extract or perfusate were mixed with 2 ml of 5 mol/l NaH_2PO_4 and shaken with 12.5 ml of ether which had been purified according to Giotti and Maynert (1951) and saturated with water. 10 ml of the ether phase was withdrawn and extracted with 2.5 ml of ether-saturated borate buffer (0.05 mol/l, pH 9.0). 1 ml of the buffer phase was taken and diluted in the case of perfusate samples with 3 ml of fresh borate buffer. An ultraviolet absorbance spectrum was recorded and the absorbance was read at the peak wavelength of 210 nm. Perchlorate extracts were treated identically

except that 1 ml of the buffer phase was diluted with only 2 ml of fresh buffer, and a second 1 ml portion of the buffer phase was diluted with 2 ml of 0.1 mol/l HCl. The absorbance at 210 nm in acid solution was subtracted from the absorbance in pH 9 buffer in order to correct for the effects of interfering substances present in the tissue. The absorbance of a 1 m mol/l solution of DMO at pH 9.0 in a 1 cm quartz cuvette was 11.0. To obtain the true intracellular DMO concentration (T), an allowance was made for DMO in the ECF and in perfusate frozen with the tissue, by applying the "perfusate contamination" correction (Section 2.3.3).

The internal pH measured by this method was independent of the external pH at the two values of external pH used:

External pH	7.39 ± 0.01	7.57 ± 0.03
Internal pH	7.07 ± 0.08	7.06 ± 0.11
(No. of hearts)	(7)	(8)

Values are means ± S.E.M.

2.3.6 Isotopic labelling of Myocardial Ammonia

Isotopically-labelled ammonia was added to the perfusion medium as ^{15}N-ammonium chloride ("Prochem", the British Oxygen Company Ltd: 95.9% enrichment). Three concentrations were used:

(1) 10 μ mol/l ammonia, 80% enrichment
(2) 160 μ mol/l ammonia, 33% enrichment
(3) 360 μ mol/l ammonia, 19% enrichment

Labelled ammonia was present from the start of perfusion. Hearts were freeze-clamped after 15 minutes perfusion, and perchlorate

extracts were made in the usual way (Section 2.3.2.5). Since facilities for stable isotope measurement were not available within the Biochemistry Department, the number of hearts which could be investigated by this technique was limited.

2.3.6.1 ^{15}N-ammonia measurement

Ammonia must be removed from substances in the sample which might interfere with the measurement of its labelling; it must also be collected in as small a volume as possible. This was achieved by passing the sample through a cation-exchange resin column (Fenton, 1962). The direct action of alkaline sodium hypobromite on the resin eluted the ammonia and converted it to nitrogen gas (Rittenberg et al., 1939), whose ^{15}N content was determined by mass spectroscopy. No ammonia was eluted from unused resin by strong alkali in a "blank" reaction.

Adsorption of ammonia. The resin (Bio-Rad AG 50W-X4, 100-200 mesh) was activated by washing with alkali followed by acid, as described by Kingsley and Tager (1970), and stored under water at 2°C until required.

Resins have been used by several workers to adsorb plasma ammonia (Fenton, 1962; Fenton and Williams, 1968; Hutchison and Labby, 1962; Miller and Rice, 1963; Forman, 1964). All authors apart from the first simply shook a convenient volume of sample with the resin, usually 1 volume of resin to 2 of sample. Using this technique, however, it was not possible to adsorb more than 60% of an ammonia sample containing 500 µg of nitrogen on to 1 ml of resin (Table 2.6). Adsorption at pH 7.0 was little different from that at pH 2.2.

pH of solution	Total NH_4^+ in solution, µg NH_4-N	NH_4^+ adsorbed to resin, µg NH_4-N	% of added NH_4^+ adsorbed to resin
2.2	5	5.0	100
	10	8.9	89
	25	17.9	72
	100	53.3	53
	250	147.7	59
	500	283	57
	1000	532	53
7.0	10	8.3	83

Table 2.6 Adsorption of ammonia to resin in the test-tube.

1 ml of activated cation-exchange resin (see text) was shaken for 1 min with 5 ml of solution, buffered at pH 2.2 (80 mmol/l citrate) or pH 7.0 (80 mmol/l phosphate), containing varying amounts of ammonium chloride. Residual ammonia was assayed in the supernatant after centrifugation, using the enzymic method.

Total ammonia added to column	=	508 µg NH_4-N
Ammonia unadsorbed on first passage through column	=	1.8 "
Ammonia eluted in 10 ml wash of column	=	2.2 "
Hence ammonia adsorbed to resin	=	504 "
Percentage of added ammonia adsorbed	=	99.2%

Table 2.7 Adsorption of ammonia to resin in a column.

To a perchlorate extract of a rat heart, prepared as in section 2.3.2.5 of this chapter, was added ammonium chloride equivalent to 500 µg of ammonia nitrogen, giving a total of 508 µg of ammonia nitrogen in a total volume of 6.0 ml. The solution was filtered and added to 1.0 ml of activated resin in a column, 0.7 cm diameter. The eluate was collected and ammonia was measured in it by the enzymic method. The column was washed with 10 ml of water, and ammonia was measured in the washing.

95.4% of the adsorbed ammonia (482 µg of ammonia nitrogen) was recovered by elution of the column with 5 ml of 4 mol/l sodium chloride.

Initial glutamate concentration, m mol/l	% of glutamate oxidized in	
	30 min	60 min
0.6	39	-
0.3	62	77
0.12	87	100

Table 2.8 Glutamate oxidation coupled to reduction of dissolved oxygen.

Reactions were carried out at 37°C in the dark, in a total volume of 10 ml. Concentrations of NAD, EDTA, GluDH and PMS were as given in the text. Initial concentrations of glutamate in the reaction mixtures were as shown. The extent of reaction was determined after 30 and 60 minutes by measurement of the ammonia formed (phenol-hypochlorite method). Solutions were not aerated during the reaction.

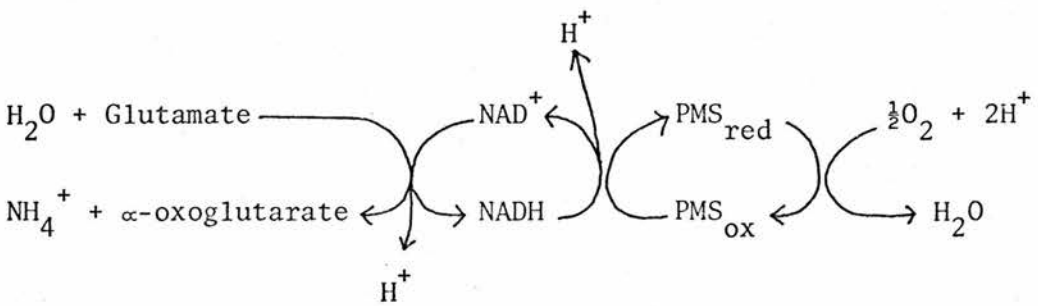


Figure 2.7 Redox events in glutamate oxidation coupled to reduction of dissolved oxygen.

However, if 1 ml of resin was poured into a column (Fenton, 1962), 99.2% of an ammonia sample containing 500 µg of nitrogen at pH 7.0 could be adsorbed on to the resin (Table 2.7).

Adsorbed ammonia could be quantitatively eluted with 4 mol/l sodium chloride, but in order to keep the volume to a minimum the resin was treated directly with alkaline hypobromite in the Rittenberg tube.

2.3.6.2 ^{15}N-Glutamate Measurement

Isotope incorporation into glutamate was determined in samples from which the ammonia had been removed by the resin technique described (Section 2.3.6.1). Glutamate nitrogen in the column eluate was liberated as ammonia by the action of glutamate dehydrogenase.

The deamination of glutamate was made irreversible by the addition of PMS to catalyse the oxidation of NADH formed in the reaction (Sowerby, 1964) by oxygen dissolved in the medium (Fig 2.7). Since the solubility of oxygen in air-saturated water at 37°C is only 0.205 m mol/l or 0.410 m-g-atoms/l, glutamate present in excess of 0.4 m mol/l cannot be completely oxidized by this method. In fact, a concentration as low as 0.12 m mol/l glutamate was necessary in order to allow complete oxidation (Table 2.8). Dilution of samples to give a suitable glutamate concentration was therefore necessary. Aeration of samples, whether carried out before or during the reaction, had no effect on the extent of glutamate oxidation. The progress of the reaction was monitored by assaying ammonia in aliquots of reaction mixture by the Berthelot method (Kaplan, 1965). The enzymic method could not be used owing to the presence of PMS, which would oxidize added NADH.

Once sufficient glutamate had been converted, the ammonia formed was adsorbed on a second resin column and treated for mass spectrometry as described in Section 2.3.6.1.

2.3.6.3 Laboratory Procedure for Determination of Labelling of Glutamate and Ammonia in the Same Sample

The mass spectrometer used required a minimum of 500 µg of nitrogen gas for operation, whereas samples contained between 2 and 60 µg of nitrogen as ammonia. Therefore 500 µg of unlabelled nitrogen (as ammonium chloride) was added to all samples before adsorption to the column, the extent of isotope dilution at this stage being known accurately.

Samples of perfusate and extract were prepared in the usual way (Section2.3.2), andthe concentrations of ammonia and glutamate in them were determined accurately. An appropriate volume of each was taken, ammonium chloride solution containing 500 µg of ^{14}N was added, and the pH was adjusted to between 5 and 6 using Johnson Universal Indicator paper. At this pH glutamate does not adsorb to the resin (Fig 2.8). The sample was run on to a column containing 1 ml of resin, 0.7 cm diameter at a rate of 1 ml per minute. The column was washed once with 10 ml of distilled water. The resin was washed quantitatively into a sample tube, and excess water was carefully pipetted off after settling. No detectible ammonia was eluted from the resin by this procedure. Resin samples were stored at -15°C until required.

If the sample was also required for ^{15}N-glutamate measurement, the eluate from the first column was retained. Its volume was kept as low as possible (less than 7.5 ml). The pH of the eluate was adjusted

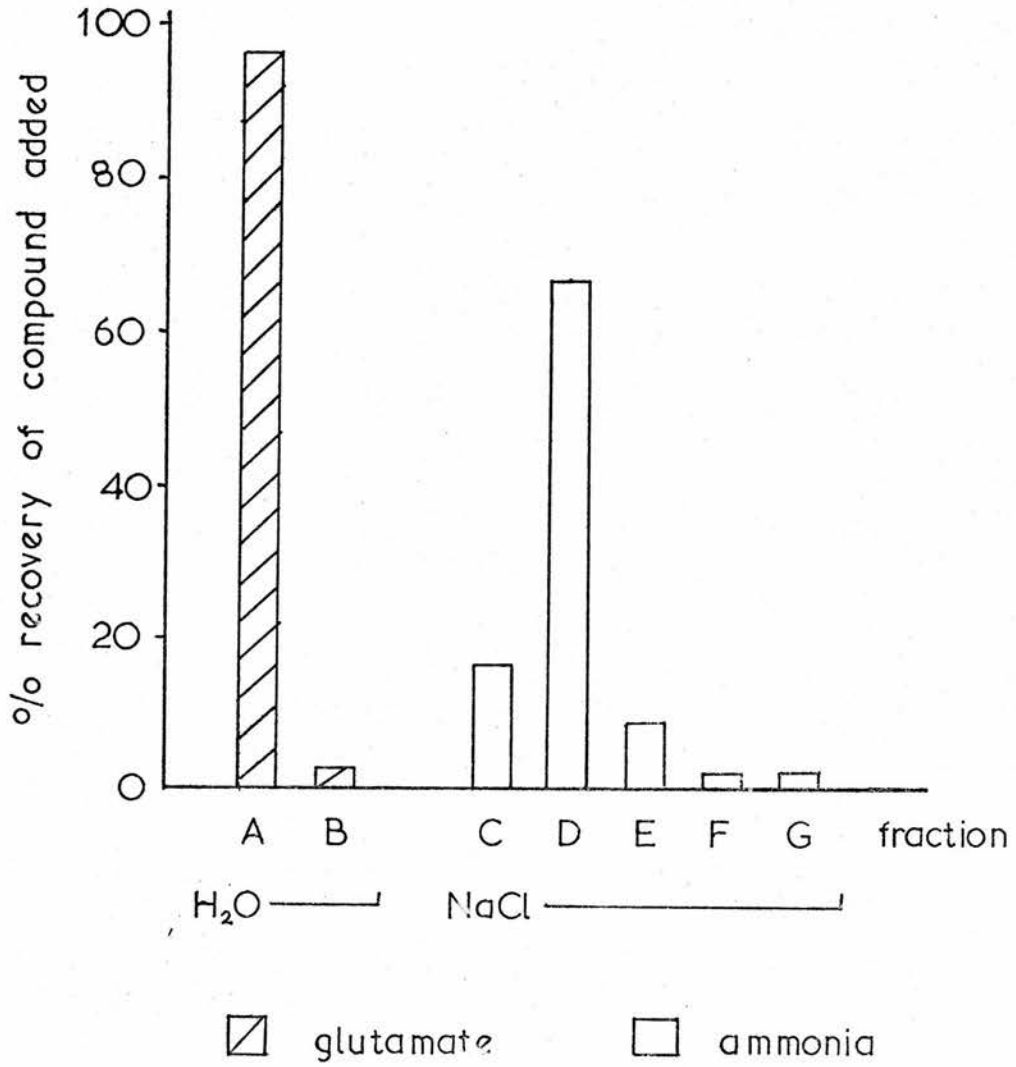


Fig. 2.8 The separation of ammonia and glutamate using a cation-exchange resin.

To a rat heart perchlorate extract were added ammonium chloride and sodium glutamate corresponding each to 500 µg of nitrogen. The pH was adjusted to 6.0 (Johnson Universal Indicator Paper) and the solution (volume 5 ml) was added to the column. The resin was washed once with 10 ml water and then with 5 ml of 4 mol/l sodium chloride. The diagram shows that all the ammonium ion is adsorbed, and can be eluted with sodium chloride, whereas all the glutamate passes through the column without adsorption. Volumes of fractions are as follows: A, 9.0 ml; B, 6.0 ml; C - G, 1.0 ml. A and B were collected before NaCl was added to the column; C - G represent elution of the column with 4 mol/l NaCl. Ammonia was measured using the enzymic method and glutamate using the hydroxylamine technique.

to 7.0 (indicator paper), and 2.0 ml of potassium phosphate buffer, pH 7.0, 0.2 mol/l was added, followed by:

NAD^+	(100 m mol/l)	0.020 ml
EDTA	(8.7 m mol/l)	0.040 ml
GluDH	(10 mg/ml in glycerol)	0.020 ml
PMS	(0.1%)	0.040 ml

The volume was adjusted to 10 ml with water, and the mixture was incubated in the dark for 45 minutes at 37°C.

At the end of the incubation, tubes were rapidly frozen in an ice-ethanol mixture to prevent further reaction and ammonia determined (Berthelot method) in an aliquot taken before freezing, to determine the extent of reaction. Tubes were heated to 90°C for 5 minutes to destroy glutamate dehydrogenase, ammonium chloride solution containing 500 µg of ^{14}N was added, and the pH was readjusted to 6.0. The mixture was run through a second column to adsorb the ammonia, the technique being exactly the same as that employed in the first adsorption.

For mass spectrometry each resin sample was washed into one leg of a Rittenberg tube. To the other leg was added 2 ml of alkaline sodium hypobromite. This was made by adding bromine dropwise to 10 mol/l NaOH until no more dissolved (Van Slyke, 1927). After evacuation of the tube the contents were mixed and the nitrogen evolved was measured in a mass spectrometer (A.E.I.). Enrichments were calculated by comparison with blanks consisting of ^{14}N-ammonia equivalent to 500 µg of nitrogen, adsorbed to 1 ml of resin.

2.4 Enzyme Localization in Rat Heart

2.4.1 Histochemical localization of enzymes

The localization of enzymes in thin sections of rat heart follows the methods described by Pette and Brandau (1966), Brandau and Pette (1966) and Sigel and Pette (1969).

2.4.1.1 Preparation of Tissue Sections

Rats were maintained on a standard laboratory diet and killed by cervical fracture and exsanguination without prior fasting. The heart was removed as quickly as possible and transferred to isotonic potassium chloride solution at $0^{o}C$; when contraction ceased the heart remained in a relaxed state. The ventricular muscle was cut into strips of 2 mm cross-section which were soaked for a further 1 minute in the KCl, rapidly blotted with filter paper, and transferred to isopentane cooled in liquid nitrogen. In one experiment a heart was perfused with ice-cold buffered KCl through the coronary system, but the tissue sections prepared from this specimen presented an identical microscopic appearance to those prepared by the standard method. The pieces of frozen tissue were mounted on a cryostat block at $-20^{o}C$, and 10 µ sections were cut with a microtome (Cryostat and microtome were products of W. Dittes, Heidelberg, W. Germany). The sections were allowed to fall on to a microscope slide and thawed by pressing a finger to the underside of the glass. They were stained as described below by the immediate application of a staining gel mounted on a cover-slip. Sections were cut, stained, fixed and mounted on the same day as removal of

the heart from the rat.

2.4.1.2 Preparation of Gel-films

Bright daylight was avoided throughout the preparation of the staining media in order to minimize the decomposition of PMS. Gel-film staining systems (Pette and Brandau, 1966) were used in the localization of all enzymes with the exception of cytochrome oxidase (Section 2.4.1.5). Agarose powder (60 mg) was mixed with 1 ml of double-distilled water and 1 ml of reaction buffer (see Section 2.4.1.4). The mixture was heated in a boiling water-bath with thorough mixing until it formed a clear solution, which was kept liquid at 42°C until required. 1 ml of the solution was withdrawn using a pre-warmed pipette with a wide orifice, and mixed with 1 ml of medium, also at 42°C, containing all the staining reagents in double concentration. The final agarose concentration was thus 1.5%.

Glass cover-slips were arranged on a metal plate heated by circulation of water at 42°C, and a 0.2 mm spacer consisting of a loop of chrome-vanadium wire was placed on each. A drop of liquid staining gel was pipetted on to the centre of each cover-slip, and a second cover-slip was placed on top of the gel and covered with a 5 g weight. The plate was cooled by passing tap-water through it, and the solidified gels were stored in a light-proof box in which water provided a humid atmosphere. Gel-slices thus prepared and stored could be used on the day of preparation, but were not kept overnight.

2.4.1.3 Staining

A suitable tissue section, containing fibres cut in longitudinal

section, was selected by viewing in the phase-contrast microscope. After removal of one of the cover-slips and the wire loop, a staining gel was inverted and placed on the tissue section on its microscope slide. The system was incubated at 37°C, observing colour development microscopically until sufficient staining had occurred. Then the gel-slice was carefully removed and the section was fixed in 4% formaldehyde and mounted in situ in glycerol-gelatine. The stained sections were photographed on the following day.

2.4.1.4 Composition of Staining Media

Test staining gels for lactate, glutamate and succinate dehydrogenases were based on the tetrazolium system of Pette and Brandau (1966); the GOT gel was developed after spectrophotometric tests in liquid media to determine the optimal reagent concentrations. In the GOT test, transamination was coupled to glutamate reduction:

$$\alpha\text{-oxoglutarate} + \text{aspartate} \xrightarrow{\text{GOT}} \text{Glutamate} + \text{oxaloacetate}$$

$$\text{Glutamate} + NADP^{+} + H_2O \xrightarrow{\text{GluDH}} \alpha\text{-oxoglutarate} + NH_4^{+} + NADPH$$

Since α-oxoglutarate is produced in the GluDH reaction, high concentrations added to the gel inhibit staining. The optimum concentration was 1.5 m mol/l. $NADP^{+}$ was used as the coenzyme in the auxiliary reaction since NADH is reoxidized by oxaloacetate and endogenous malate dehydrogenase. Glutamate dehydrogenase used as the auxiliary enzyme was freed from ammonium sulphate by overnight dialysis against 2 changes of 200 m mol/l imidazole buffer containing 5 m mol/l EDTA, pH 7.6.

The composition of the staining gels is set out below; for each enzyme control gels without substrate were also prepared.

Lactate dehydrogenase. TEA* = 50 m mol/l, pH 7.6; EDTA = 1 m mol/l; Nitro-BT = 1.5 m mol/l; NAD^+ 2.3 m mol/l; L-lactate = 24 m mol/l; DMS = 0.3 m mol/l; agarose = 1.5% (w/v).

Glutamate dehydrogenase. TEA = 50 m mol/l, pH 7.6; EDTA = 5 m mol/l; Nitro-BT = 1.5 m mol/l; NAD^+ = 2.3 m mol/l; L-glutamate = 50 m mol/l; ADP = 2 m mol/l; PMS = 0.15 m mol/l; agarose = 1.5%.

Succinate dehydrogenase. Phosphate buffer = 100 m mol/l, pH 7.6; EDTA = 5 m mol/l; Nitro-BT = 1.5 m mol/l; PMS = 0.15 m mol/l; succinate = 80 m mol/l; KCN = 1 m mol/l; agarose = 1.5%.

Glutamate-oxaloacetate transaminase. TEA = 50 m mol/l, pH 7.6; EDTA = 5 m mol/l; Nitro-BT = 1.5 m mol/l; $NADP^+$ = 5 m mol/l; α-oxoglutarate = 1.5 m mol/l; L-aspartate = 50 m mol/l; GluDH = 100 units/ml; ADP = 2 m mol/l; PMS = 0.2 m mol/l; agarose = 1.5%.

2.4.1.5 Cytochrome Oxidase

Cytochrome oxidase was localized using the diaminobenzidine (DAB) technique of Seligman et al., (1968). A liquid staining medium was used, composed as follows:

Sodium phosphate = 0.05 mol/l, pH 7.4; DAB = 0.5 mg/ml; catalase = 2 µg/ml; cytochrome c = 1 mg/ml; sucrose = 75 mg/ml (= 0.22 mol/l); total volume = 5 ml.

DAB was obtained as the tetrahydrochloride which was neutralized before use. Control gels contained in addition 10 m mol/l potassium cyanide

* A list of abbreviations can be found on p. 32.

to inhibit cytochrome oxidase. Colour development in control sections was not completely prevented unless tissue slices were preincubated in a medium containing buffer, 75 mg/ml sucrose and 10 m mol/l KCN (Nir and Seligman, 1971).

Sections were prepared on microscope slides as described in Section 2.4.1.1, and 100-200 µl of staining medium was pipetted on to each. After 40 minutes incubation at 25°C the section was carefully blotted, fixed in 5% acetic acid, dried and mounted in glycerol-gelatine.

2.4.1.6 Exogenous Alcohol Dehydrogenase

In experiments in which the possible adsorption of exogenous alcohol dehydrogenase to the muscle fibres was studied, a section was prepared by the standard technique (Section 2.4.1.1) and a 5 µl drop of a test solution was placed upon it by pipette. Two test solutions and two control solutions were used:

1. Alcohol dehydrogenase, 10 mg/ml in 0.8 mol/l ammonium sulphate.
2. Control: 0.8 mol/l ammonium sulphate.
3. Alcohol dehydrogenase, 10 mg/ml in 2 m mol/l phosphate, pH 7.0.
4. Distilled water control.

Alcohol dehydrogenase was obtained as a 30 mg/ml suspension in 2.4 mol/l ammonium sulphate; solution (1) was prepared from it by dilution, and solution (3) by dialysis against 2 m mol/l phosphate buffer followed by volume adjustment.

After 3 minutes at room temperature, excess liquid was carefully soaked away from the tissue section with filter paper and a staining gel was applied. Colour development was sufficient after 5 minutes incubation at 37°C. Sections were photographed immediately,

with the gel-slice in position, since the ADH incubation technique loosens the tissue from the slide and renders removal of the gel impossible. In a further control experiment a 5 µl drop of 15 m mol/l NADH solution was applied to the tissue section, in an analogous manner.

The gel-film staining media employed in these experiments had the following composition:

Exogenous ADH: TEA = 50 m mol/l, pH 7.6; EDTA = 1 m mol/l; Nitro-BT = 1.5 m mol/l; NAD^+ = 3.0 m mol/l; Ethanol = 44 m mol/l; PMS = 0.15 m mol/l; agarose = 1.5%.

Exogenous NADH: TEA = 50 m mol/l, pH 7.6; EDTA = 5 m mol/l; Nitro-BT = 1.5 m mol/l; PMS = 0.15 m mol/l; agarose = 1.5%.

2.4.2 Enzyme Localization in Disrupted Tissue

2.4.2.1 Fractional Extraction of Endogenous Enzymes

The technique used was that of Pette (1968). Rats were maintained on a standard diet (Oxoid 41B), and were killed by cervical fracture and exsanguination without prior fasting. The heart was removed immediately, cut into small pieces and minced at 2°C in a specially-constructed micro-meat-mincer (B. Braun, Apparatebau, Melsungen, W. Germany). 500 mg of minced tissue was weighed into a 10 ml centrifuge tube and suspended in 9.5 ml of sucrose medium (0.3 mol/l sucrose, 10 m mol/l triethanolamine hydrochloride, 2 m mol/l EDTA, adjusted to pH 7.4). The suspension was stirred for 15 minutes in an ice-bath by a teflon plumper moving vertically at 80 cycles per minute. The clearance between the plumper and the sides and

bottom of the tube was sufficiently wide that shearing forces in the liquid, which might cause rupture of organelles, were avoided. After 15 minutes the tube was centrifuged for 20 minutes at 140,000 g (Spinco L50 ultracentrifuge, rotor type 50 Ti, 45,000 rpm) and the clear supernatant was decanted and labelled S_1. Resuspension of the pellet in sucrose medium and repetition of the procedure yielded supernatant S_2. Fractions S_1 and S_2 represent the "washing out" of the cytoplasmic compartment by a medium of low ionic strength without liberating enzymes from mitochondria. The pellet was resuspended in phosphate buffer (0.1 mol/l, pH 7.2), "plumpered" for 15 minutes and recentrifuged to yield supernatant S_3. This medium causes swelling of the mitochondria and rupture of the outer membrane with liberation of intermembranal enzymes such as adenylate kinase from liver mitochondria (Pette, 1966). The pellet was homogenized in 2 ml of phosphate buffer (Polytron PT 10 homogenizer, Kinematic, Luzern, Switzerland), diluted to 10 ml with phosphate buffer and stirred for 10 minutes. Centrifugation yielded supernatant S_4 and the pellet P, which was resuspended in 0.05 mol/l phosphate, pH 7.2, using a ground-glass hand-homogenizer. S_4 contains enzymes soluble in the intramitochondrial matrix; P contains membrane-bound enzymes.

Enzyme activities in all fractions were measured spectrophotometrically at 25°C by standard reactions involving appearance or disappearance of NADH (Bücher et al., 1964); succinate dehydrogenase was measured by the reduction of cytochrome C by succinate in the presence of cyanide. Enzymes in fractions containing mitochondrial membranes (e.g. fraction P) were measured after inhibition of NADH oxidase by the addition of rotenone (2 µl of a 0.5 m mol/l solution in ethanol) to the cuvette.

2.4.2.2 Adsorption test for ADH in a heart homogenate

A freshly-excised rat heart was washed for 10 minutes by coronary perfusion with ice-cold Krebs medium (Krebs and Henseleit, 1932), the temperature effect on pH being overcome by altering the bicarbonate concentration. 0.5 g of tissue was homogenized (Ultraturrax homogenizer, Janke and Kunkel) in a medium of low ionic strength (0.3 mol/l sucrose, 5 m mol/l triethanolamine hydrochloride, 0.5 m mol/l magnesium sulphate, pH = 7.2). Yeast alcohol dehydrogenase was added (0.1 mg, having an activity of 8.40 μ moles per minute at 25°C as measured by the Racker (1950) assay), and the solution was diluted to 10 ml with sucrose medium. The mixture was stirred at 0°C for 20 minutes to permit any adsorption to take place, and centrifuged at 36,000 g for 25 minutes (Spinco L50 ultracentrifuge, rotor type 40, 40,000 rpm) to yield the supernatant S_1. The sediment was resuspended and washed for 15 minutes with a second 10 ml portion of sucrose medium to yield supernatant S_2. The pellet from this step was resuspended and washed in a medium of high ionic strength (0.1 mol/l phosphate, pH 7.2), yielding supernatant S_3 and the pellet P, which was resuspended in phosphate buffer. Alcohol dehydrogenase was measured in all fractions by the method of Racker (1950), with the addition of semicarbazide and glutathione in the concentrations recommended by Boehringer and Soehne (Biochimica catalogue). Rotenone was added before assaying the enzyme in the pellet fraction (see Section 2.4.2.1).

Appendix 2.I

A specimen preparation of Glutaminase from Pig's kidney

Klingman and Handler (1958)

Sowerby and Ottaway (1966)

Kidneys from freshly-killed pigs (slaughterhouse), total 227 g, were homogenized using a Waring blendor in 5 volumes (1,135 ml) of 8.5% (w/v) sucrose, containing 0.02 mol/l borate, pH 8.1. The homogenate was centrifuged at 1,500 g for 15 minutes (3,000 rpm in the 6 x 250 ml rotor of an MSE High Speed 18 centrifuge) and the sediment was discarded. The supernatant was strained through two layers of muslin and centrifuged to precipitate the mitochondria (20,000 g, 20 min). The sediment was lyophilized.

The dried powder (14.23 g) was homogenized at -10°C for 2 minutes (Waring Blendor) in 504 ml of redistilled butanol, pre-cooled to -10°C. The homogenate was sucked almost to dryness on a Buchner funnel, rehomogenized for 30 seconds at -10°C in 229 ml of acetone redistilled from potassium permanganate, and returned to the suction funnel. Just before dryness, 50 ml of redistilled diethyl ether was added to the powder in the funnel, which was then sucked dry. The powder was dried overnight in a vacuum desiccator.

To prepare a soluble extract containing the enzyme, the dry powder (weight 11.92 g) was stirred for 1 hour in 286 ml of 0.01 mol/l borate, pH 8.1 at 4°C. The suspension was centrifuged at 29,000 g for 15 minutes (MSE High Speed 18, 6 x 100 ml rotor, 15,000 rpm). 119 ml of cold 4 mol/l potassium phosphate, pH 8.0, was added to the supernatant dropwise over a 30 minute period, stirring continuously.

The suspension was stirred for a further 15 minutes and centrifuged at 29,000 g, and the precipitate was dissolved in 47.6 ml of 0.01 mol/l borate, pH 8.1. The enzyme was precipitated a second time with 23.8 ml of 4 mol/l phosphate, spun down and redissolved in the minimum volume (18 ml) of a buffer containing 0.03 mol/l borate and 0.02 mol/l phosphate, pH 8.1. The solution was dialyzed overnight against 2 litres of the same buffer, changing the buffer once, to remove protein-bound glutamate. A precipitate formed during dialysis, which was not observed by Sowerby and Ottaway (1966). After centrifugation, however, virtually all the enzyme activity remained in the soluble supernatant, which was dispensed in 2½ ml portions into glass vials and stored at -15°C. The frozen enzyme was stable for 6 months without serious loss of activity, but was rapidly inactivated by repeated freezing and thawing. Enzyme solution in regular use was therefore diluted with an equal volume of Analar glycerol and stored at -15°C. Under these conditions the enzyme remained active for at least one month.

The enzyme was assayed during its preparation. The rate of production of ammonia from glutamine by the enzyme was measured using excess glutamate dehydrogenase in the following mixture:

Sodium phosphate buffer, 0.2 mol/l, pH 7.2	0.500 ml
NADH, 8.5 mg/ml	0.020 ml
α-Oxoglutarate, 150 m mol/l	0.050 ml
GluDH, Boehringer solution in glycerol (10 mg/ml)	0.040 ml
Aliquot of glutaminase preparation	
Glutamine, 0.2 mol/l	0.050 ml
Distilled water to a total volume of	1.000 ml

All adducts except glutamine were mixed in the cuvette. Once all endogenous ammonia had disappeared (absorbance steady), the glutamine

was added and the initial rate of absorbance decrease was recorded. Table 2.9 shows the progress of the glutaminase purification. The protein content of the solution was estimated by measuring the absorbance at 280 nm and 260 nm (Warburg and Christian, 1941) using an SP 500 spectrophotometer (Pye-Unicam, Cambridge). The glutaminase preparation contained, finally, 24 mg of protein per ml, or 12 mg/ml after dilution with glycerol.

Sample material assayed	Total Volume ml	Total Glutaminase, µmol/min at 37°C	Total Protein, g	Specific activity, µmol/min/g protein
Mitochondrial precipitate before lyophilization	190	76.3	8.89	86
Extracted butanol powder	300	82.8	3.59	231
Final 4 mol/l phosphate precipitate, before dialysis	18	181.1	0.62	292
Ditto, after dialysis	28.5	338.0	0.62	546
Supernatant after centrifuging dialysate	25.0	371.0	0.61	603
Pellet after centrifuging dialysate	8.9	15.1	Not measured	-

Table 2.9 The activity of glutaminase during preparation.

Activity was measured as described in the text. The apparent increase in the total glutaminase activity during preparation is due to the removal of substances inhibiting the assay.

CHAPTER 3

SOME KINETIC STUDIES ON AMMONIA IN PERFUSED RAT HEART

Chapter 3. Some Kinetic Studies on Ammonia in Perfused Rat Heart.

3.1 Ammonia in Animal Tissues.

It was suggested (Chapter 1, Section 1.4) that the relatively high ammonia concentration in tissues such as muscle may be of physiological importance. The concentration of ammonia was measured in three tissues of the rat and compared with values reported by other authors (Table 3.1). The concentration of ammonia is similar in all the tissues shown in Table 3.1. Some workers reported values higher than these, which can usually be attributed to one of two causes. Early workers (e.g. Meyerhof et al., 1925; Embden et al., 1928b) did not appreciate that dying or traumatized tissue produces large amounts of ammonia (Parnas and Mozolowsky, 1927), and allowed too long a time to elapse between removal of the tissue and making the extract. Some more recent workers, who are primarily concerned with amino acid concentrations (e.g. Manchester and Wool, 1963; Scharff and Wool, 1965a), quote a value corresponding to the ammonia peak eluted by an amino acid analyzer, which is generally high owing to the presence of ammonium salts in the reagents used.

The concentration of ammonia in plasma is much lower than that in muscle tissue. Representative data are given in Table 3.2. Larger values than these are probably again due to less specific methods of analysis (Chapter 2, Section 2.2.7). Concentrations in whole blood show greater variation than do plasma concentrations. Erythrocytes have a high internal ammonia concentration (Seligson and Hirahara, 1957). Furthermore, erythrocytes possess adenylate deaminase (Dimond, 1955; Lowenstein, 1972). Action of this enzyme

Tissue	Ammonia concentration, µmol/g fresh weight	Reference and Notes
		Present Study
Rat liver	0.632, 0.470	Single determinations, chemical method
Rat leg muscle	0.410	
Rat heart	0.430 ± 0.030	Enzymic method, mean ± S.E.M. of 6 hearts
		Other Authors
Frog muscle	0.825	Embden et al., 1928b
Frog muscle	0.356	Parnas and Mozolowski, 1927
Pigeon thigh-muscle	0.570	
Mouse hind-leg	0.785	
Rabbit leg muscle	0.250	
Rat leg muscle	0.274	Gerez and Kirsten, 1965
Pigeon breast muscle	0.342	
Locust leg muscle	0.455	
Locust flight muscle	0.282	
Rat heart	0.419	Stewart et al., 1969

Table 3.1 Ammonia concentration in animal tissues.

Source of blood	Ammonia Concentration mmol/l	Method of Assay	Reference
Human plasma	0.028	Microdiffusion	Brown et al., 1957
Human plasma	0.046	"	Seligson and Hirahara, 1957
Human whole blood	< 0.004	GluDH	Kirsten et al., 1963
Human plasma	0.029	"	Mondzac et al., 1965
Human plasma	0.026	"	Rubin and Knott, 1967
Human whole blood	0.032	"	Muting et al., 1968
Human plasma	0.012	Ion-exchange	Fenton, 1962
Human plasma	0.018	"	Fenton and Williams, 1968
Rabbit whole blood	0.074	Microdiffusion	Reif, 1960
Rat whole blood	0.030	"	Astakhova and Golubev, 1967

Table 3.2 Concentration of ammonia in mammalian blood, as given by various authors.

on AMP is probably responsible for the rapid increase in the ammonia content of whole blood after drawing (Scudder and Smith, 1940; Reif, 1960; Mondzac et al., 1965). Inhibition of the enzyme by heparin may result in the lower values characteristic of heparinized blood (Dimond, 1965). The failure of some workers to demonstrate any ammonia in the blood of rested, fasting humans (Kirsten et al., 1963) is at variance with the majority of authors. The ammonia concentration in rat blood is 0.03 m mol/l (Astakhova and Golubev, 1967).

A concentration of ammonia inside a muscle cell is thus 20 to 30 times greater than that in the plasma. It will be shown (Section 3.1.1) that a rat heart perfused with a medium of ammonia concentration similar to that of plasma maintains a similar concentration ratio. These results seem to conflict with the view that ammonia is freely diffusible across membranes (cf Jacobs, 1940). In the present chapter the kinetics of ammonia production and uptake by the perfused rat heart are described, and possible mechanisms for maintaining the high concentration ratio are discussed.

3.1.1 Ammonia in the Perfused Rat Heart

Table 3.3 shows the ammonia content of rat hearts, before and after perfusion. Hearts were perfused for 75 minutes at 37°C and pH 7.39 with Krebs-Henseleit medium supplemented with glucose (see Chapter 2, Section 2.3.2.2). During this time, ammonia was produced by the heart and released into the perfusate. After 75 minutes the external ammonia concentration was similar to that in blood (Astakhova and Golubev, 1967), and the ratio of tissue concentration to perfusate concentration resembled that found in vivo. There was

little net change in the concentration of ammonia in the tissue. This situation could be maintained up to the end of 2 hours perfusion without further change (Section 3.2.1).

	Ammonia concentration, mmol/l,		Ratio Tissue/Perfusate
	Tissue	Perfusate	
Before perfusion	0.86 ± 0.06	0.007 ± 0.001	-
After perfusion	0.64 ± 0.08	0.031 ± 0.003	21
In vivo	0.86 ± 0.06	0.03 (Blood)	29

Table 3.3 Ammonia concentrations in the perfused rat heart.

Concentrations of ammonia, measured in perfusate samples and in perchlorate extracts of tissue as described in the text, are means ± S.E.M. Tissue concentrations are calculated as mmol/l intracellular water, assuming the volume of the ICF is approximately 0.5 ml/g tissue (Scharff and Wool, 1965a). The ammonia concentration of rat blood is that given by Astakhova and Golubev, 1967.

3.2 Changes in Ammonia Concentration on Perfusion of Rat Heart

3.2.1 The Metabolism of Ammonia by Perfused Rat Heart

It has been shown (Table 3.3) that hearts perfused at pH 7.39 with Krebs-Henseleit medium produce detectable traces of ammonia. In order to determine whether a balance could be reached between output and uptake of ammonia, hearts were perfused with media to which increasing concentrations of ammonium chloride had been added. Table 3.4 and Fig 3.1 show ammonia lost or gained by the whole system during perfusion ("change in total ammonia"), which represents ammonia synthesized or utilized in heart metabolism. The change in perfusate ammonia accounts for the major part of the change in total ammonia, the change in tissue ammonia being relatively small. Thus the change in perfusate ammonia reflects the metabolism of ammonia by the heart rather than a redistribution of ammonia across the membrane.

Net uptake of ammonia from media of standard pH and potassium concentration did not occur unless the initial perfusate concentration exceeded 210 µmol/l. The requirement for a high external concentration before uptake will occur has also been shown in frog muscle, which does not take up ammonia unless the external concentration exceeds 700 µmol/l (Netter, 1934). Hearts were also perfused with media of non-standard pH or potassium concentration in connection with investigations to be discussed later in this chapter. For simplicity the metabolic events associated with these media will be presented here alongside those results obtained with standard media. Net uptake from media of high ammonia concentration was less if the pH of the perfusate was increased to 7.57. However, reduction of the extracellular potassium concentration promoted ammonia uptake at

Conditions	Initial Perfusate ammonia concentration mmol/l	Final tissue ammonia concentration, µmol/g	Change in ammonia concentration on perfusion, µmol/g heart	
			Perfusate	Total
Unperfused	-	0.43 ± 0.03 (6)	-	-
Perfused, pH 7.39 K^+ normal	0.007 ± 0.001	0.32 ± 0.04	+1.56 ± 0.18	+1.45 ± 0.25 (9)
	0.076 ± 0.006	0.41 ± 0.07	+2.04 ± 0.49	+2.02 ± 0.56 (4)
	0.205 ± 0.015	0.52 ± 0.02	+0.28 ± 0.13	+0.36 ± 0.14 (2)
	0.307 ± 0.013	0.77 ± 0.07	-3.87 ± 0.38	-3.54 ± 0.32 (3)
Perfused, pH 7.57 K^+ normal	0.009 ± 0.001	0.35 ± 0.06	+1.56 ± 0.50	+1.47 ± 0.46 (6)
	0.076 ± 0.004	0.51 ± 0.08	+1.05 ± 0.41	+1.13 ± 0.43 (5)
	0.136 ± 0.018	0.54 ± 0.07	+1.69 ± 1.01	+1.80 ± 1.08 (2)
	0.184 ± 0.012	0.45 ± 0.08	+1.46 ± 1.07	+1.17 ± 1.41 (3)
	0.312 ± 0.011	0.68 ± 0.06	-0.18 ± 0.74	-0.20 ± 0.88 (6)
Perfused, pH 7.39 K^+ x 0.6	0.011 ± 0.002	0.49 ± 0.09	+2.24 ± 0.44	+2.29 ± 0.44 (4)
	0.066 ± 0.005	0.15 ± 0.02	-2.35 ± 0.52	-2.63 ± 0.51 (3)
	0.116 ± 0.002	0.33 ± 0.03	-0.60 ± 0.43	-0.70 ± 0.45 (3)
	0.206 ± 0.007	0.43 ± 0.07	-0.64 ± 1.65	-0.64 ± 1.68 (3)
	0.330 ± 0.018	0.56 ± 0.08	+0.20 ± 1.77	+0.34 ± 1.78 (4)

Table 3.4 Changes in ammonia concentration on perfusion of rat heart.

Ammonia was measured in tissue and perfusate, before and after perfusion, as described in the text. Values are means ± S.E.M., with the number of hearts in parenthesis. Where only two hearts were analyzed, the value is given as mean ± range. The total ammonia change in the system is computed as the sum for each heart of perfusate change + tissue change. The tissue change is calculated as the tissue concentration after perfusion for each heart — the mean concentration in unperfused heart (0.43 µmol/g).

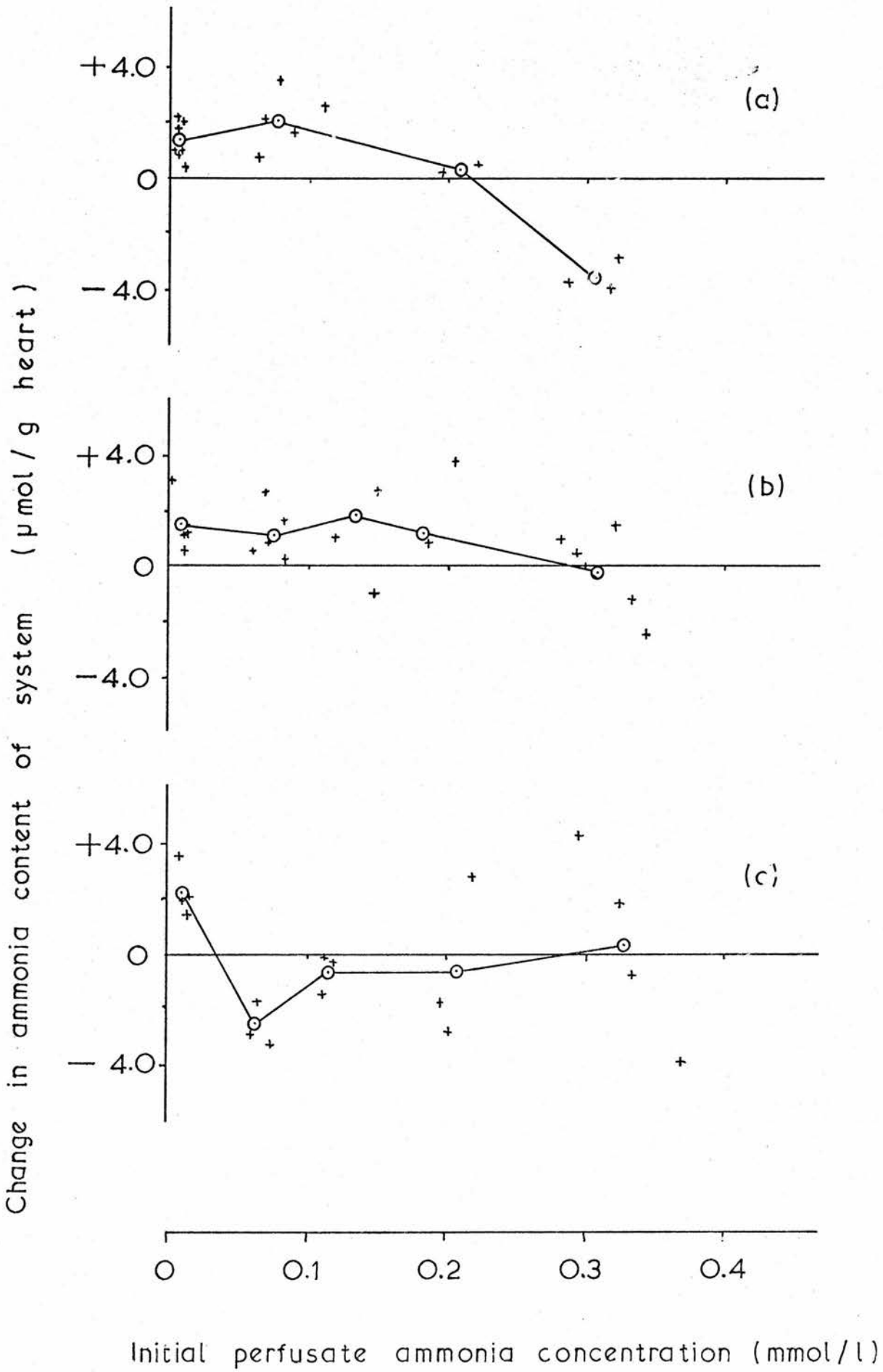
Fig. 3.1 Changes in ammonia concentration on perfusion of rat heart with media of varying initial ammonia concentration.

The graphs show the gain or loss of ammonia in the system (tissue + perfusate) due to metabolism, as μmol/g fresh weight. The data are tabulated in table 3.4. Crosses represent individual hearts; circles are means of groups of hearts perfused with media of the same initial ammonia concentration.

(a) pH 7.39, K^+ normal

(b) pH 7.57, K^+ normal

(c) pH 7.39, K^+ 0.6 x normal.

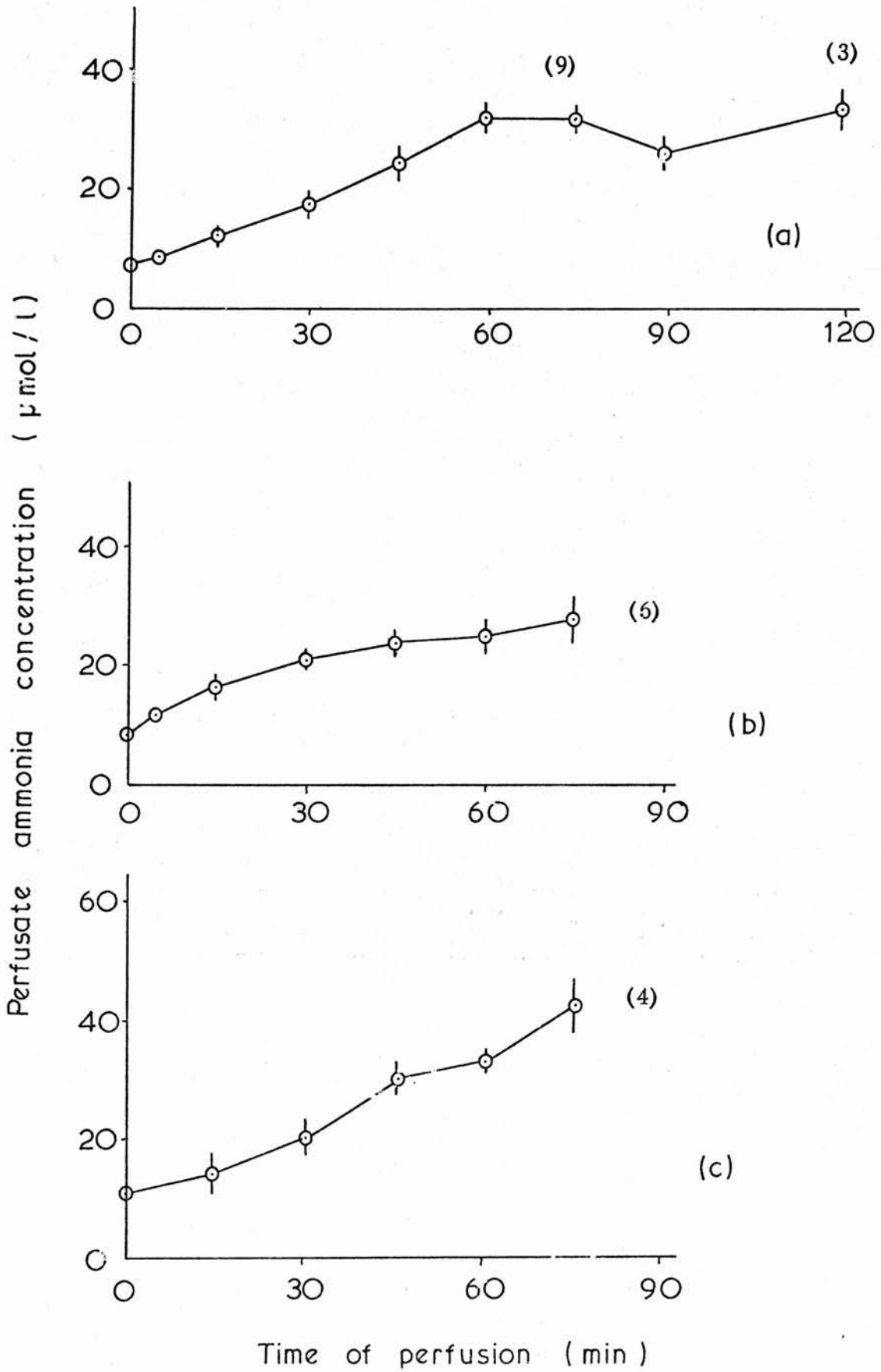

+4.0
0
−4.0
(a)
+4.0
0
−4.0
(b)
+4.0
0
−4.0
(c)
Change in ammonia content of system (μmol / g heart)
0
0.1
0.2
0.3
0.4
Initial perfusate ammonia concentration (mmol/l)

intermediate initial ammonia concentrations. The ammonia balance in hearts perfused with low external potassium and high external ammonia was not reproducible.

No irregularities in beating or outward appearance of the tissue were observed in any heart at any time during perfusion with the higher concentrations of ammonia, nor was any decrease in oxygen consumption seen (Chapter 2, Table 2.5).

3.2.2 The Time-Course of Ammonia Changes in Perfusate

The efflux of ammonia into perfusing fluid to which no ammonia has been added is shown in Fig 3.2. The initial concentration of 7.1 µmol/l represents ammonium salts present as contaminants in the components of the Krebs-Henseleit medium. Production is approximately linear for the first 60 minutes, at a rate which is comparable to the rate of output of alanine and glutamine (Chapter 5, Section 5.2.3.4). The ammonia concentration in the perfusate reaches a maximum of 31 µmol/l after 60 minutes, which is similar to the concentration in blood (Astakhova and Golubev, 1967), and remains constant for a further 60 minutes of perfusion. There are two possible explanations for this phenomenon. Ammonia synthesis in the tissue may cease altogether at the end of 60 minutes. However, it seemed more likely that a dynamic equilibrium exists after 60 minutes in which the rate of ammonia production equals the rate of its utilization. The finding that hearts can take up ammonia from media of high ammonia concentration supports the latter hypothesis, as do subsequent experiments with ^{15}N-labelled ammonia (Chapter 4). A similar maximum concentration was reached in perfusion with the medium of pH 7.57, whereas efflux into the


40
20
0
(9)
(3)
(a)
0
30
60
90
120
Perfusate ammonia concentration (μ mol / l)
40
20
0
(5)
(b)
0
30
60
90
60
40
20
0
(4)
(c)
0
30
60
90
Time of perfusion (min)

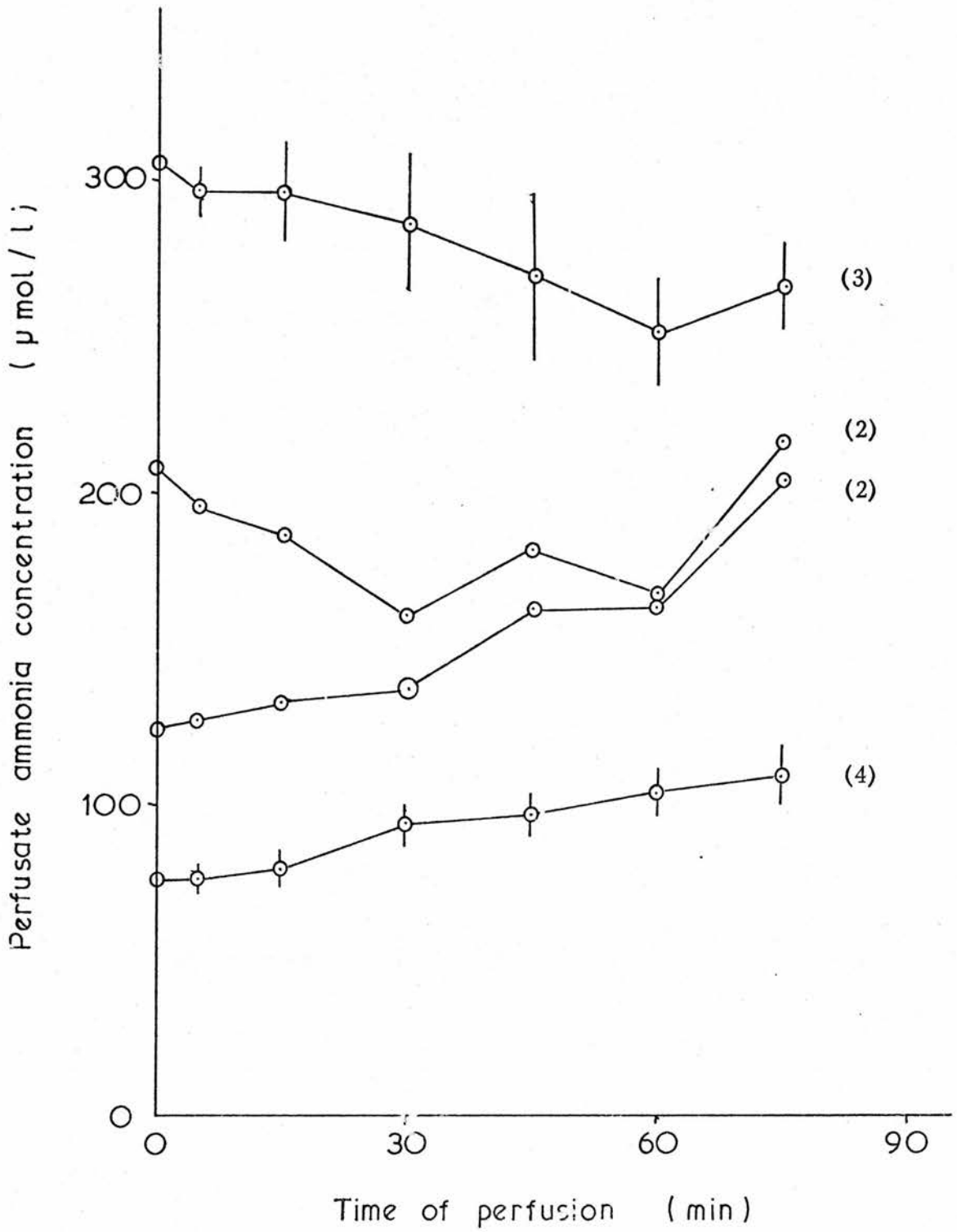


Fig. 3.3 The time-course of ammonia in perfusate, pH 7.39, K^+ normal.

Ammonia was measured in serial samples of perfusate; the initial concentration was varied by addition of ammonium chloride to the perfusing medium.

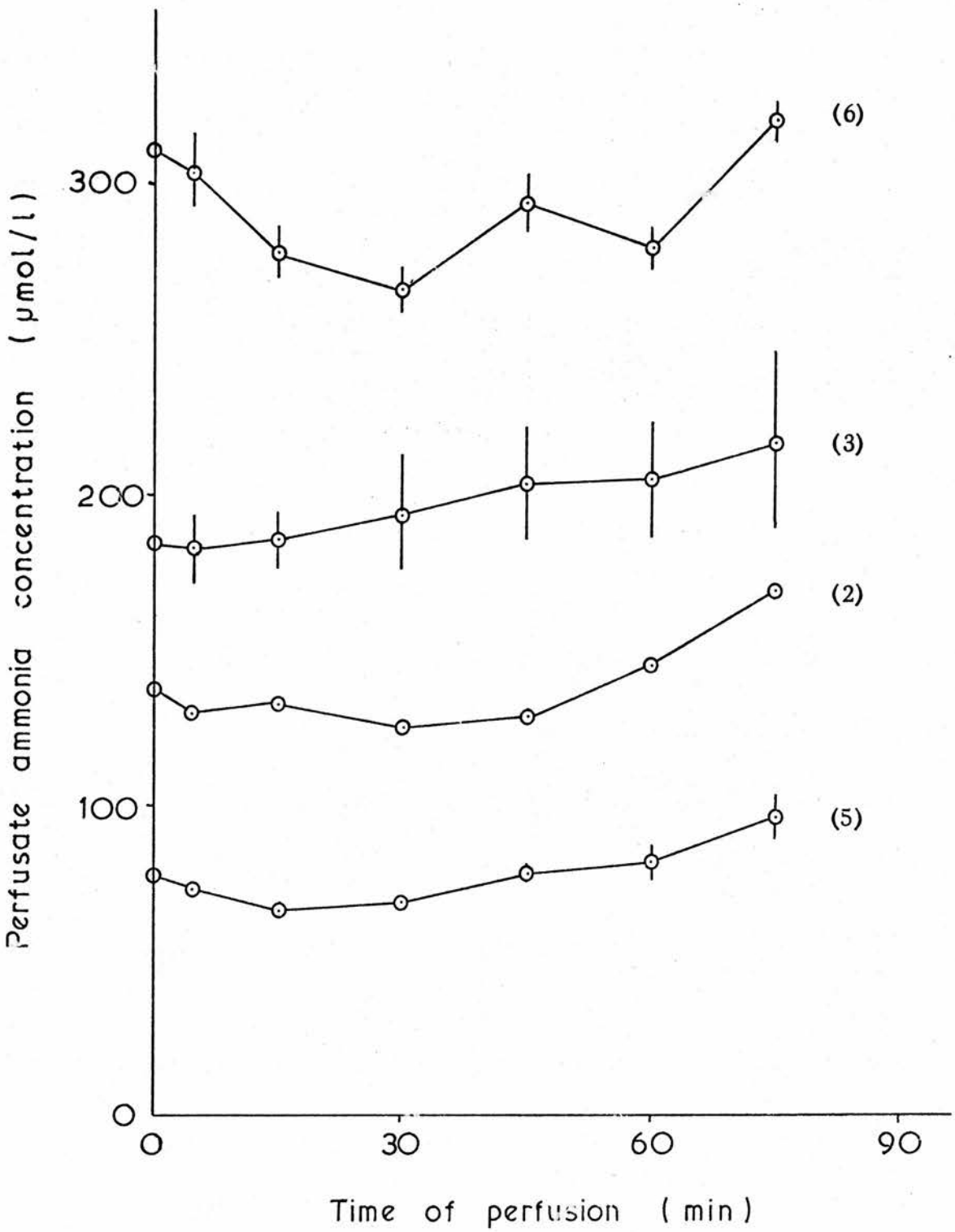


Fig. 3.4 The time-course of ammonia measured in perfusate, pH 7.57, K^+ normal.

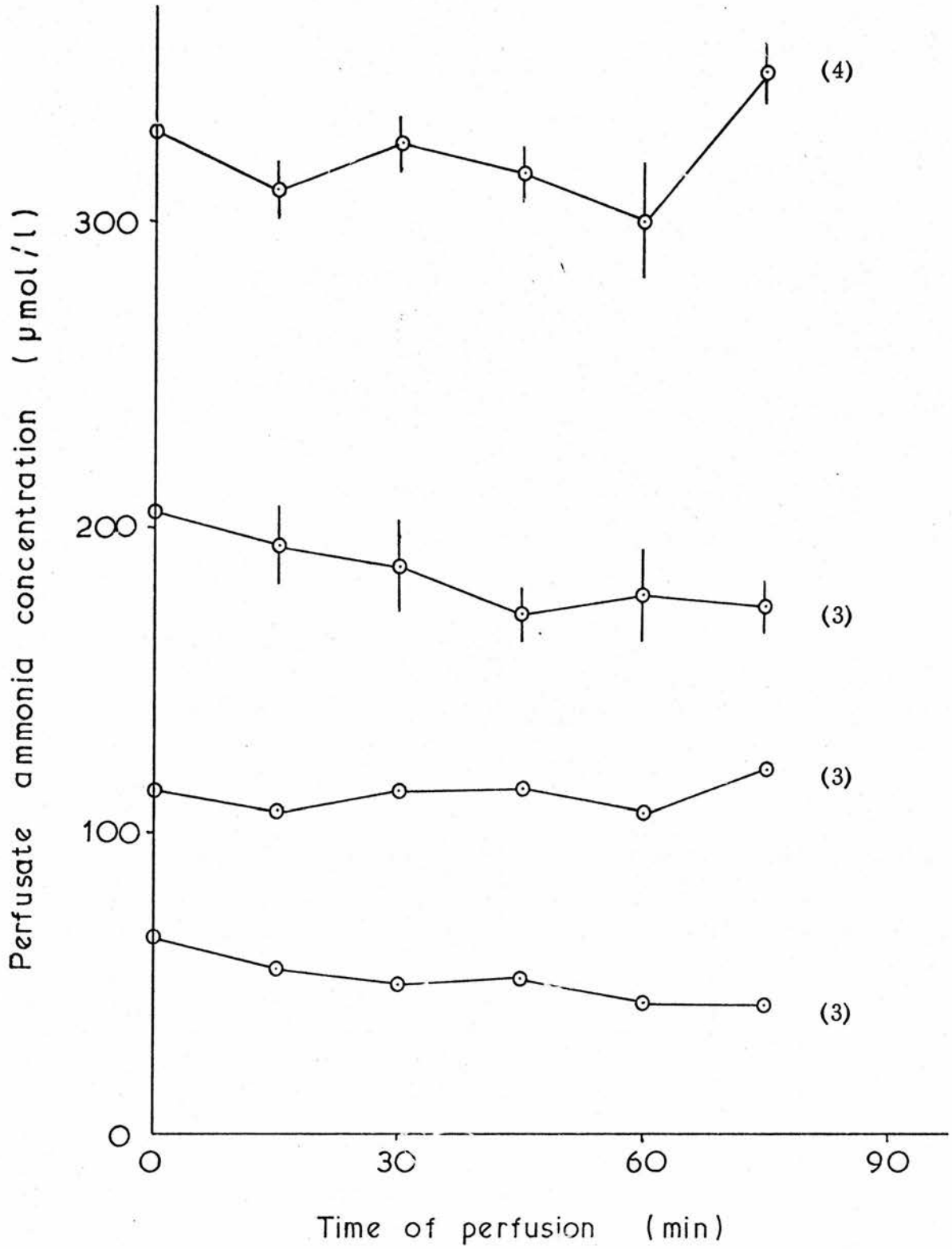


Fig. 3.5 The time-course of ammonia measured in perfusate, pH 7.39, K^+ 0.6 x normal.

low-potassium medium continued throughout 75 minutes.

Changes in ammonia concentration at higher initial concentrations are shown in Fig 3.3. If the initial concentration was above 150 µmol/l it tended to fall during perfusion and vice versa. A tendency of the ammonia concentration in the perfusate of some hearts to rise in the final 15 minutes may signify incipient heart failure due to the unphysiological ammonia concentration, and was not seen in hearts perfused at pH 7.39 with concentrations of ammonia similar to those of plasma (see Chapter 5, Section 5.3.5). Uptake from media of high initial concentration was relatively slow, and there was no net uptake after 60 minutes. The perfusate concentration never actually fell to the plasma level.

The effects of high pH and low potassium on these time-courses are shown in Figs 3.4 and 3.5.

3.2.3 The Time-Course of Ammonia Changes in Tissue

Tissue ammonia in routine perfusion was only measured at the end of 75 minutes. However, a few hearts were perfused for different periods, and the tissue ammonia concentration in these is given in Table 3.5.

The data of Table 3.5 suggests that tissue ammonia rises in the first 15 minutes of perfusion, falling later to a concentration similar to that in the unperfused heart. However, the 15 minute values were from the hearts used in ^{15}N-labelling studies, and it is possible that the high tissue ammonia values measured in these hearts are due to the effects of impurities in the ^{15}N-ammonium

chloride (see Chapter 4, Section 4.1). The data provide no evidence for any further significant change in tissue ammonia after 75 minutes.

Initial perfusate ammonia concentration, mmol/l	Time of perfusion, min	Tissue ammonia concentration, μmol/g
Unperfused	0	0.43 ± 0.03 (6)
0.007	15	0.89, 1.35
	75	0.32 ± 0.04 (9)
	90	0.42
	120	0.60, 0.30
0.310	15	2.25, 0.54
	75	0.77 ± 0.07 (3)

Table 3.5 Effect of perfusion time on tissue ammonia concentration.

Ammonia was measured in the tissue, before and after perfusion. Values are means ± S.E.M., where the number of hearts appears in parenthesis. Other values are the concentrations in individual hearts.

3.3 The Distribution of Ammonia across the Heart Cell Membrane

It has been considered by many authors, whose opinions have already been presented (Chapter 1, Section 1.6) that ammonia diffuses freely across biological membranes of widely differing kinds, such as those of erythrocytes, aquatic organisms, gut cells and mitochondria. In general, investigations in higher animals suggest diffusion of uncharged ammonia rather than diffusion of ammonium cation. If this were true also of the heart cell membrane, one would expect the ammonia concentration inside the tissue to be approximately twice that outside (Section 3.3.1.1). The measurement of ammonia concentrations in rat heart and blood demonstrates the much higher ratio of 30 : 1, and a ratio of 20 : 1 was found in the isolated perfused heart (Table 3.3). In the present section possible mechanisms of ammonia transport are investigated and discussed.

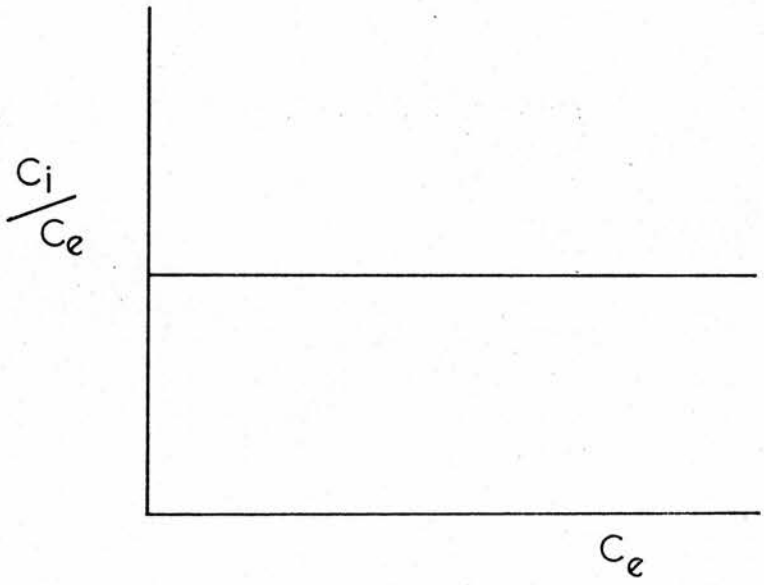
3.3.1 The Expected Ammonia Distribution

If ammonia diffuses freely across the heart cell membrane, whether as uncharged ammonia or as ammonium cation, one would expect the ratio of the ammonia concentration inside to that outside to remain constant at all values of external concentration (Fig 3.6a). Thus the internal ammonia concentration (Ci) should be directly proportional to the external concentration (Ce), (Fig 3.6b).

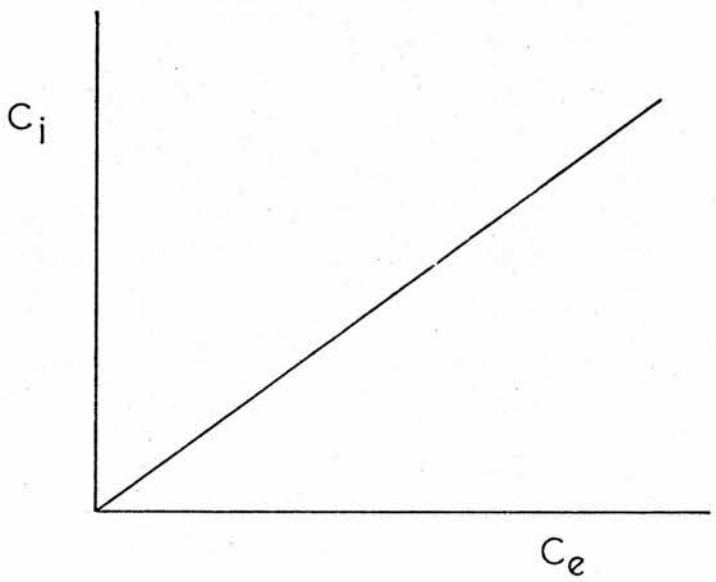
It should theoretically be possible by further tests to distinguish whether NH_3 or NH_4^+ is the permeant species.

3.3.1.1 Free diffusion of uncharged ammonia

Since the proportion of free NH_3 in a mixture of NH_3 and NH_4^+



(a)



(b)

Fig. 3.6 The expected ammonia distribution.

depends on the pH of the solution, the distribution of ammonia in this case will depend on the pH gradient across the membrane and can be predicted mathematically (Jacobs, 1940; Stabenau et al., 1959) as follows:

$$Ci/Ce = \frac{1 + 10^{(pKa - pHi)}}{1 + 10^{(pKa - pHe)}} \quad \ldots\ldots\ldots (1)$$

where Ci and Ce are the concentrations of ammonia in the intracellular and extracellular fluid respectively; pHe is the extracellular pH; pHi is the intracellular pH; and pKa is the acid dissociation constant of ammonium ion. Under physiological conditions pKa = 9.03 (Klocke et al., 1972); pHi = 7.07 at both values of perfusate pH tested (Chapter 2, Section 2.3.5). Hence at a perfusate pH of 7.39, Ci/Ce = 2.06 and at a perfusate pH of 7.57, Ci/Ce = 3.09. Thus the ratio Ci/Ce will increase with the alkalinity of the perfusate (Stabenau et al., 1959), as a result of the increase in the concentration of uncharged ammonia in the external medium. Ci will still be linearly related to Ce (Fig 3.7a).

3.3.1.2 Free diffusion of ammonium cation

Earlier authors assumed that the membrane potential was influenced only by the potassium ion distribution, and that the distribution of potassium and other ions was related to the membrane potential by the Nernst equation, i.e.

$$e^{-\frac{F}{RT}} = \frac{[K^+]i}{[K^+]e} = \frac{[NH_4^+]i}{[NH_4^+]e} \quad \ldots\ldots (2) \quad \text{(see Conway and Moore, 1945)}$$

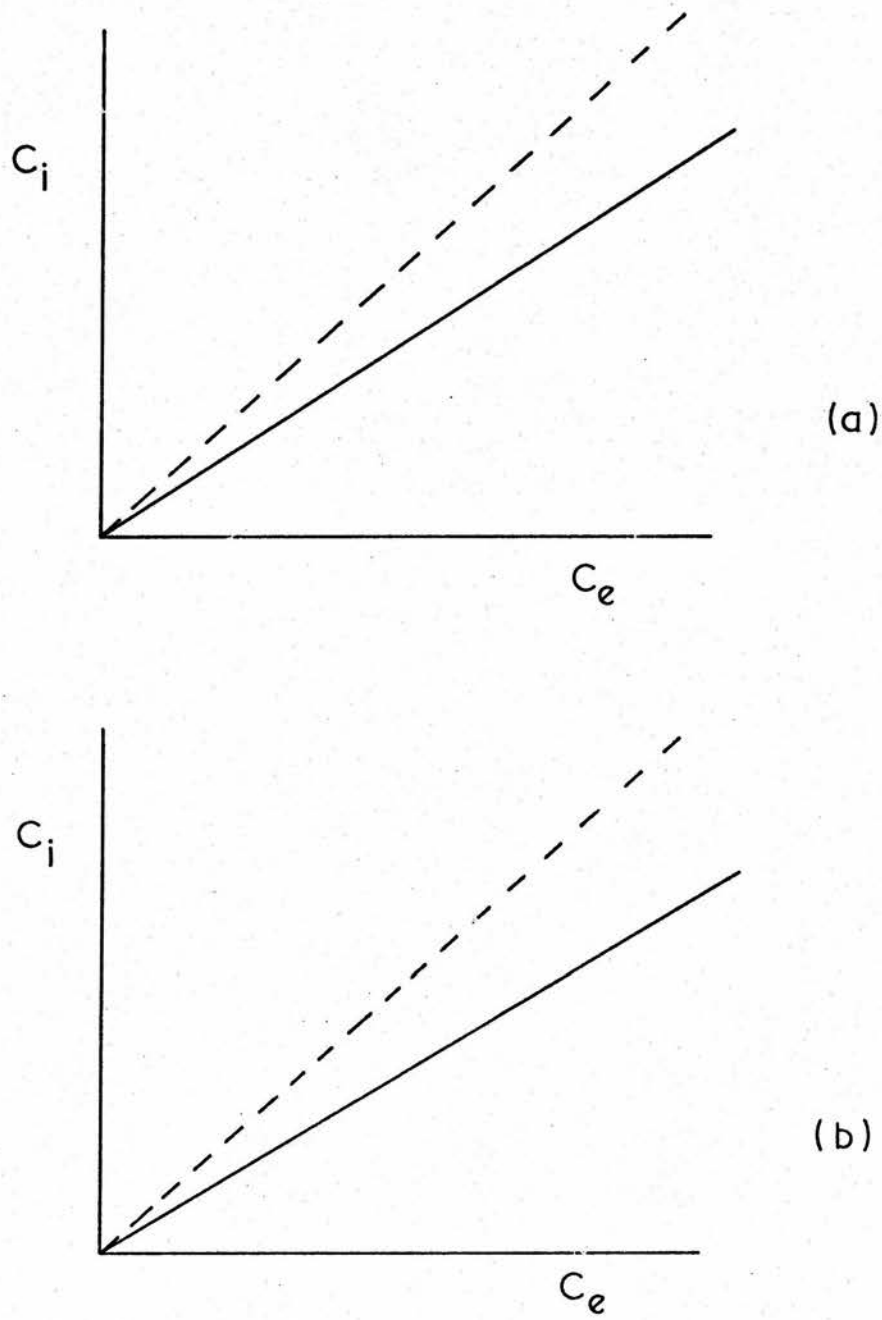


Fig. 3.7 Expected ammonia distribution,

(a) NH_3 permeant:
Solid line: normal pH
Dotted line: raised pH.

(b) NH_4^+ permeant:
Solid line: normal $[K^+]$
Dotted line: decreased $[K^+]$.

Hence the expected value for $\frac{[NH_4^+]_i}{[NH_4^+]_e}$ (or Ci/Ce), as for $\frac{[H^+]_i}{[H^+]_e}$, was approximately 40. The value found for ammonia in frog muscle was much lower than this (Conway and Moore, 1945; Netter, 1934).

It is now appreciated that the membrane potential is a function of all permeant ions present (Goldman, 1944; Ginsborg, 1973), e.g.

$$E = \frac{RT}{F} \ln \frac{P_k K_e + P_{Na} Na_e + P_{Cl} Cl_i + P_{NH4} NH4_e + \ldots}{P_k K_i + P_{Na} Na_i + P_{Cl} Cl_e + P_{NH4} NH4_i + \ldots} \quad \ldots\ (3)$$

Where P_A is a permeability constant for the ion A with extracellular and intracellular concentrations (more correctly activities) A_e and A_i respectively. Since the membrane is more permeable to potassium than to other ions ($P_k > P_{Na}$, etc.), the membrane potential is effectively determined by the ratio of potassium concentrations across it. Since $K_i > K_e$, the interior of the cell is negative with respect to the exterior.

When the potassium concentration of the extracellular fluid is reduced, some of the intracellular potassium tends to leak into the ECF tending to restore the equilibrium. However, *in vivo* this leakage is very slow (Fenn *et al.*, 1945), requiring several days (Muntwyler *et al.*, 1950; Sarsione and Muntwyler, 1965), and the proportional reduction in the potassium concentration in the tissue is never so great as that in the serum (Muntwyler *et al.*, 1950). Hence in a short-term experiment reduction of the perfusate potassium concentration does not significantly affect the tissue potassium, and is an effective means of increasing the membrane potential (Adrian, 1956).

If ammonia diffuses as NH_4^+ cation, its distribution will depend

on the membrane potential, but the ratio Ci/Ce need not have the value of 40 as determined by the Nernst equation. The actual value of the ratio cannot be predicted from equation (3) without accurate knowledge of the value of P_{NH4} and the membrane potential. Moreover, a tissue such as heart muscle which exhibits continuous electrical activity does not have a constant membrane potential. Nevertheless, altering the membrane potential by decreasing K_e/K_i ought to cause a decrease in the ratio NH_{4e}/NH_{4i}. Furthermore, NH_{4e} will still be linearly related to NH_{4i} (Fig 3.7b), provided that $P_{NH4} \times NH_{4i} << P_k K_i$ and $P_{NH4}.NH_{4e} << P_k K_e$; these conditions are fulfilled in all the perfusions to be described.

3.3.1.3 Summary

Given the premise that one form of ammonia diffuses freely, the following will be true. If uncharged ammonia is the permeant species, raising the perfusate pH should cause an increase in Ci/Ce, but altering the potassium concentration should have no effect (Fig 3.7a). The simple picture of Fig 3.7a will only be adhered to provided that ammonia diffusion does not alter the internal pH. If ammonia cation is the permeant species, decreasing the extracellular potassium concentration should bring about an increase in Ci/Ce, whereas altering the pH should have no effect (Fig 3.7b).

3.3.2 The observed ammonia distribution in perfused rat heart

3.3.2.1 The effect of perfusate ammonia concentration on the ammonia distribution

The concentration of ammonia in tissue and perfusate was measured

in rat hearts after perfusion for 75 minutes with Krebs medium in which the initial ammonia concentration was varied. The results are shown in Table 3.6. Plotting tissue concentration (Tμ moles per g fresh weight) against perfusate concentration (P μ moles per ml) shows that T is linearly related to P, but that the best straight line does not pass through the origin (Fig 3.8). The best straight line was calculated by computer, using a method which allowed for error of measurement in both T and P. The values of slope and intercept were:

Intercept (A) = 0.25 μ moles per g fresh weight
Slope (m) = 1.71 ml per g fresh weight

T μ moles per g and P μ moles per ml are the quantities measured experimentally. T and P thus have different dimensions, but in this way no premature assumptions are made with respect to the volume accessible to the tissue ammonia. If this volume (v ml per g fresh weight) is known, then the true ammonia concentration (Ci) in the intracellular fluid is related to the measured quantity T as follows:

$$Ci = T/v \ \mu \text{ moles per ml}$$

Assuming that there is no permeability barrier between the perfusate and the extracellular fluid, the symbols P and Ce are interchangeable.

3.3.2.2. The effect of perfusate pH on the ammonia distribution

Dependence of the ammonia distribution on pH was tested by altering the pH of the perfusing fluid. A medium of more alkaline pH was chosen, nearer to the pKa of ammonia (Klocke et al., 1972), in

order that a relatively small pH change might achieve the maximum effect on the degree of dissociation of the ammonium ion. A pH of 8 or higher tends to cause precipitation of calcium salts (Krebs and Henseleit, 1932) and also affects glycolysis (Kloppick et al., 1967), and was therefore avoided. A pH of 7.57 was used, approximately 0.2 units above that of the standard medium. This was achieved by increasing the bicarbonate concentration of the perfusate (Krebs and Henseleit, 1932; see Chapter 2, Section 2.3.2.2).

Hearts perfused with this medium continued to beat normally and showed no change in outward appearance. The initial concentration of ammonia in the medium was varied and ammonia was measured in tissue and perfusate at the end of perfusion (Table 3.7). Tissue ammonia was plotted against perfusate ammonia concentration (Fig 3.9), and a best straight line was drawn. The values of slope and intercept were:

Intercept (A) = 0.36 μ moles per g
Slope (m) = 1.02 ml per g

3.3.2.3 The effect of perfusate potassium concentration on the ammonia distribution

Hearts were perfused with a medium in which the potassium concentration had been reduced by 40%, by replacing half of the potassium chloride of Krebs Ringer by sodium chloride. The hearts continued to beat normally and showed no change in outward appearance. The ammonia concentrations in tissue and perfusate at the end of perfusion are shown in Table 3.8. Plotting tissue ammonia against perfusate ammonia (Fig 3.10) gives slope and intercept as

follows:

Intercept	(A)	=	0.07 μ moles per g
Slope	(m)	=	1.84 ml per g

Legend to Tables 3.6 - 3.8

The ammonia concentration in tissue and perfusate of each heart was measured at the end of 75 minutes perfusion as described in the text. Tables 3.6 - 3.8 show the values for individual hearts, together with mean values (+ standard error of mean) for groups of hearts perfused with media of the same initial ammonia concentration.

The initial ammonia concentration was varied by adding ammonium chloride to the perfusing fluid. Values given in the table are means of concentrations measured in the medium at the start of each perfusion.

The pH and potassium concentration of the perfusing fluid were varied as described in Chapter 2, Section 2.3.2.2.

Mean initial perfusate ammonia concentration, µmol/ml	Heart weight, g	Final perfusate ammonia concentration, µmol/ml (P)	Final tissue ammonia concentration, µmol/g (T)	Distribution ratio, ml/g (T/P)
0.007	0.72	0.058	0.43	7.4
	0.93	0.032	0.29	9.0
	0.88	0.021	0.43	20.6
	0.77	0.043	0.42	9.6
	0.60	0.021	0.27	13.1
	0.63	0.033	0.19	5.5
	0.63	0.064	0.44	7.0
	0.59	0.026	0.15	5.8
	0.60	0.034	0.23	6.7
Mean ± S.E.M.		0.031 ± 0.005	0.32 ± 0.04	9.4 ± 1.6
0.076	0.80	0.084	0.28	3.3
	0.74	0.107	0.42	3.9
	0.70	0.119	0.37	3.2
	0.66	0.130	0.58	4.4
Mean ± S.E.M.		0.110 ± 0.010	0.41 ± 0.07	3.7 ± 0.3
0.124	0.64	0.159	0.23	1.4
	0.66	0.250	0.37	1.5
Mean		0.204	0.30	1.5
0.205	0.72	0.235	0.53	2.3
	0.63	0.197	0.50	2.5
Mean		0.216	0.52	2.4
0.307	0.67	0.272	0.84	3.1
	0.76	0.286	0.63	2.2
	0.65	0.240	0.83	3.5
Mean ± S.E.M.		0.266 ± 0.014	0.77 ± 0.07	2.9 ± 0.4

Table 3.6 Effect of perfusate ammonia concentration on ammonia distribution. (pH = 7.39, K^+ normal)

Mean initial perfusate ammonia concentration, µmol/ml	Heart weight, g	Final perfusate ammonia concentration, µmol/ml (P)	Final tissue ammonia concentration, µmol/g (T)	Distribution ratio, ml/g (T/P)
0.009	0.64	0.021	0.60	28.2
	0.63	0.027	0.30	11.2
	0.69	0.029	0.42	14.6
	0.64	0.022	0.28	13.0
	0.59	0.057	0.33	5.7
	0.53	0.041	0.17	4.0
Mean ± S.E.M.		0.033 ± 0.006	0.35 ± 0.06	12.8 ± 3.5
0.076	0.65	0.107	0.68	6.4
	0.67	0.089	0.70	8.0
	0.73	0.115	0.30	2.6
	0.97	0.096	0.38	4.0
	0.55	0.071	0.51	7.1
Mean ± S.E.M.		0.095 ± 0.007	0.51 ± 0.08	5.6 ± 1.0
0.136	0.76	0.202	0.61	3.0
	0.63	0.134	0.47	3.5
Mean		0.168	0.54	3.3
0.184	0.73	0.276	0.46	1.7
	0.81	0.210	0.32	1.5
	0.63	0.143	0.58	4.0
Mean ± S.E.M.		0.210 ± 0.038	0.45 ± 0.08	2.4 ± 0.8
0.312	0.74	0.308	0.57	1.8
	0.65	0.314	0.46	1.5
	0.83	0.354	0.81	2.3
	0.83	0.296	0.67	2.3
	0.79	0.314	0.82	2.6
	0.70	0.326	0.77	2.3
Mean ± S.E.M.		0.319 ± 0.008	0.68 ± 0.06	2.1 ± 0.2

Table 3.7 Effect of high perfusate pH on ammonia distribution.
(pH = 7.57, K^+ normal)

Mean initial perfusate ammonia concentration, µmol/ml	Heart weight, g	Final perfusate ammonia concentration, µmol/ml (P)	Final tissue ammonia concentration, µmol/g (T)	Distribution ratio, ml/g (T/P)
0.011	0.67	0.041	0.67	16.5
	0.64	0.053	0.54	10.2
	0.74	0.034	0.50	14.7
	0.69	0.042	0.23	5.5
Mean ± S.E.M.		0.043 ± 0.004	0.49 ± 0.09	11.7 ± 2.5
0.066	0.51	0.045	0.15	3.4
	0.50	0.037	0.18	4.9
	0.56	0.050	0.11	2.2
Mean ± S.E.M.		0.044 ± 0.004	0.15 ± 0.02	3.5 ± 0.8
0.116	0.68	0.120	0.39	3.2
	0.63	0.122	0.31	2.6
	0.56	0.103	0.29	2.8
Mean + S.E.M.		0.115 + 0.006	0.33 + 0.03	2.9 + 0.2
0.206	0.76	0.165	0.50	3.0
	0.70	0.185	0.29	1.6
	0.70	0.269	0.51	1.9
Mean ± S.E.M.		0.206 ± 0.032	0.43 ± 0.07	2.4 ± 0.3
0.330	0.70	0.334	0.73	2.2
	0.71	0.366	0.46	1.3
	0.66	0.364	0.64	1.8
	0.72	0.330	0.42	1.3
Mean ± S.E.M.		0.349 ± 0.009	0.56 ± 0.08	1.7 ± 0.2

Table 3.8 Effect of low perfusate potassium concentration on ammonia distribution. (pH = 7.39, K^+ = 0.6 x Normal).

Figs. 3.8 - 3.10: The observed ammonia distribution.

The quantity of ammonia in the heart tissue (T μmol/g) is plotted against the concentration of ammonia in the perfusate (P mmol/l). Both quantities were measured after perfusion for 75 min. Crosses represent individual hearts; circles are the means (± S.E.M., except where mean of 2) of groups of hearts perfused with media of the same initial ammonia concentration. The line of best fit was calculated by computer (see text); in this calculation the four hearts perfused with low-potassium medium without addition of ammonia, which behaved anomalously (see discussion), were omitted. The data are tabulated in Tables 3.6 -3.8.

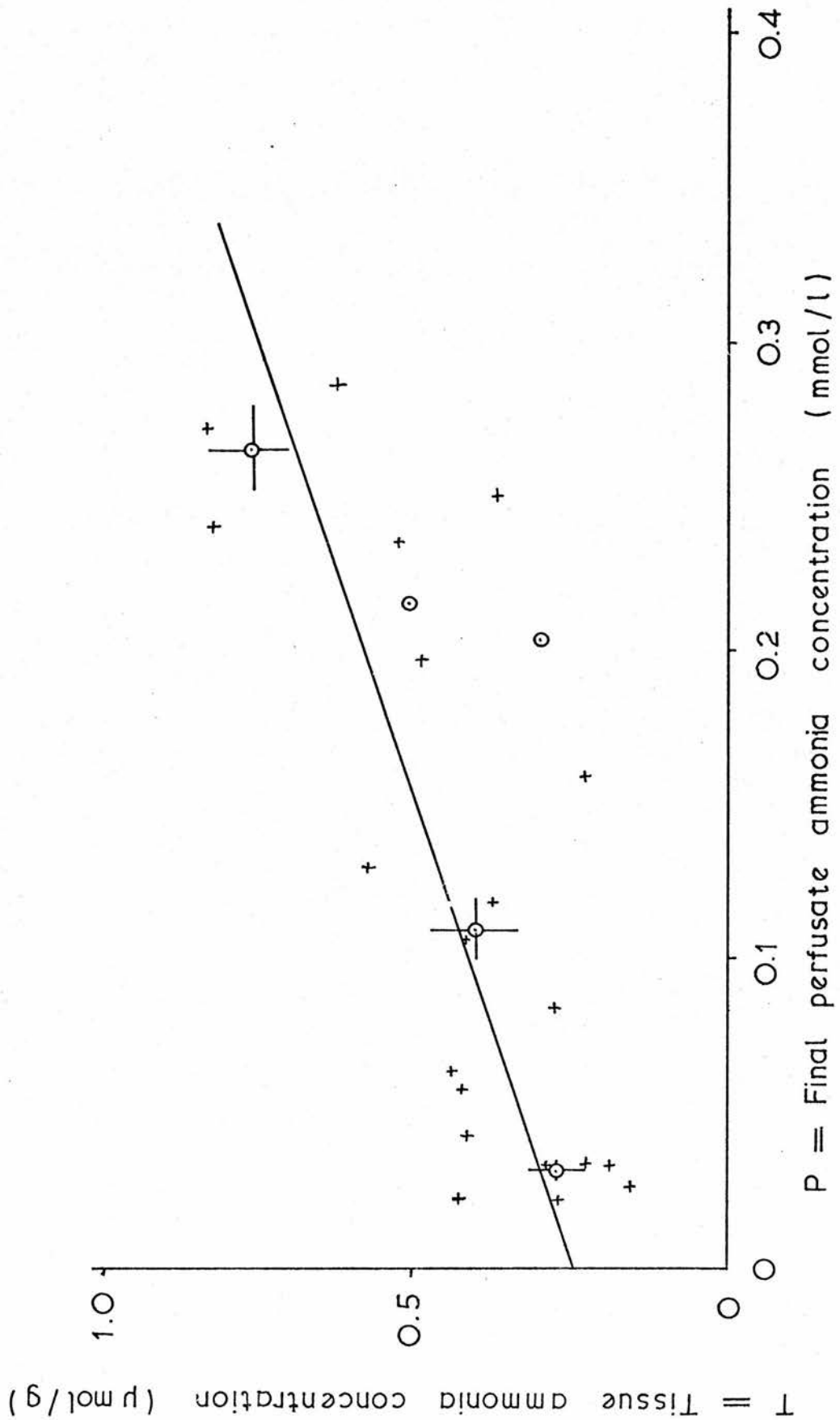


Fig. 3.8 The observed distribution of ammonia between tissue and perfusate, pH 7.39, K^+ normal.

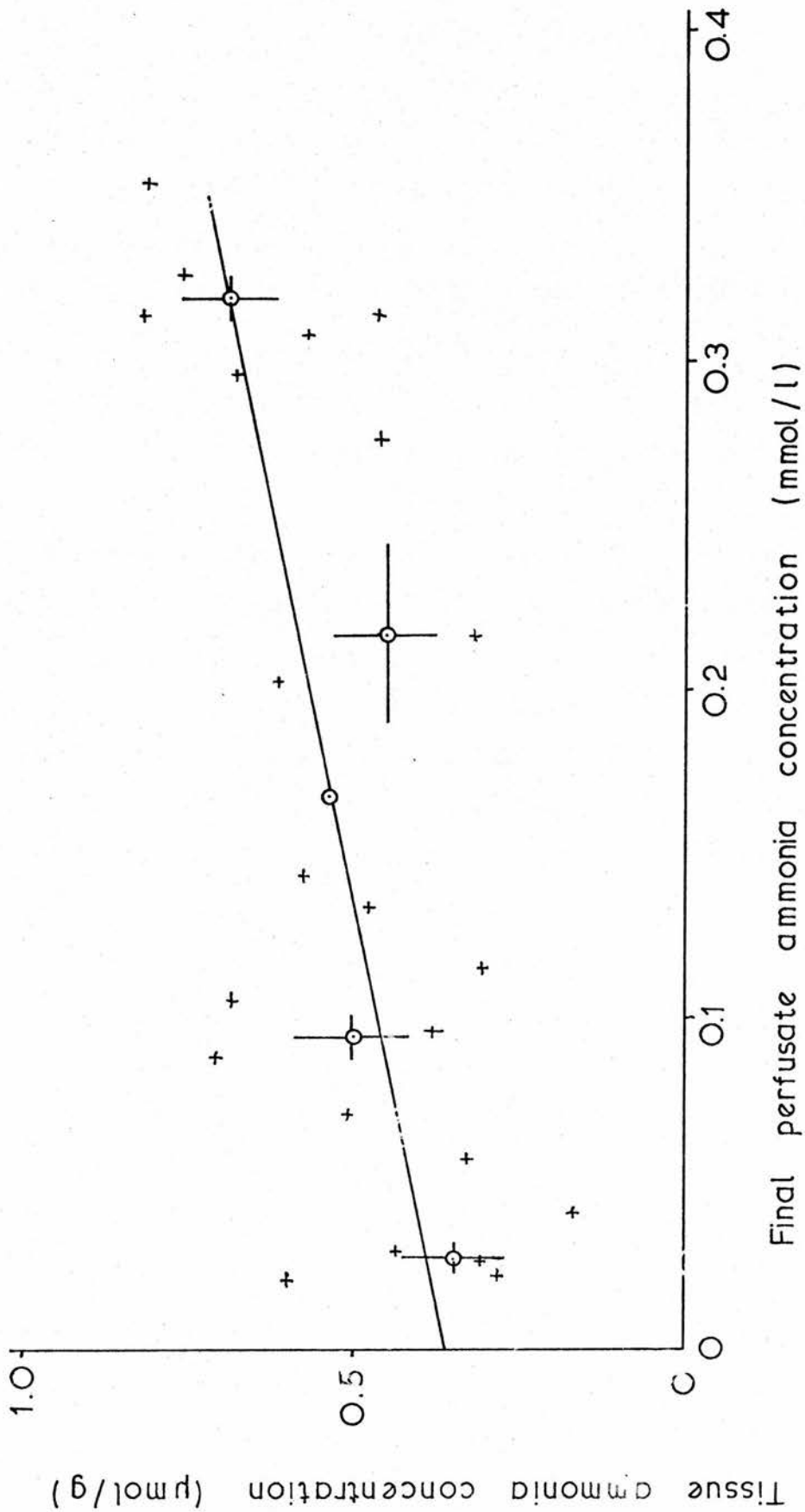


Fig. 3.9 The observed distribution of ammonia between tissue and perfusate, pH 7.57, K^+ normal.

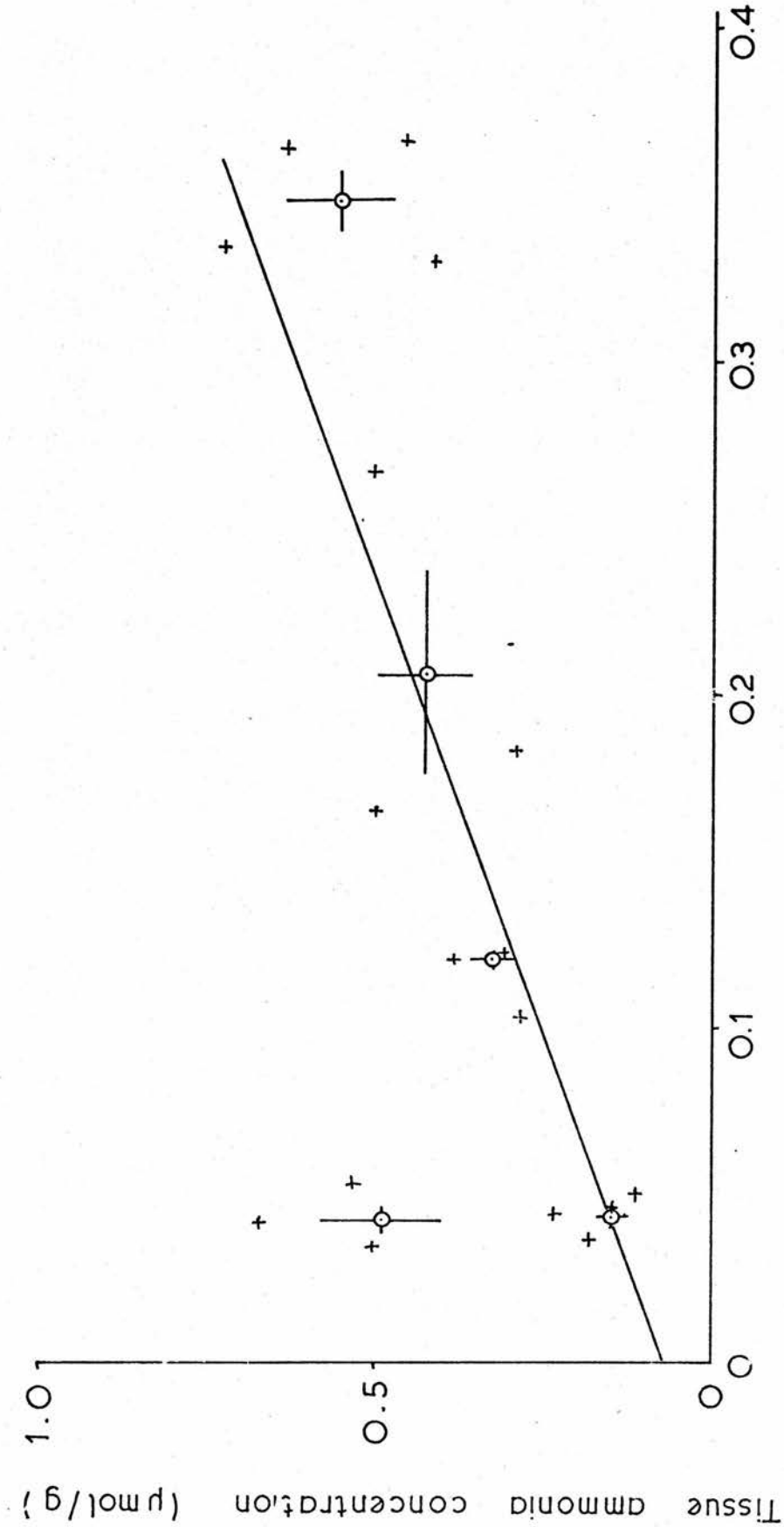


Fig. 3.10 The observed distribution of ammonia between tissue and perfusate, pH 7.39, K^+ 0.6 x normal.

3.4 Dicussion

3.4.1 The ammonia distribution

If one species of ammonia is freely diffusible, the expected ratio of ammonia concentration inside the cell to that outside (T/P) should be independent of the external concentration whether the permeant species is uncharged ammonia or ammonium cation (Fig 3.6a). Fig 3.11 shows that this is not the case in the perfused rat heart. The ratio T/P, approximately 10 in the heart perfused without added ammonia, falls to a limiting value of about 2.6 as the external concentration rises. If it is assumed that the internal ammonia fills the intracellular space irrespective of its concentration in the extracellular fluid, these figures are equivalent to a fall in Ci/Ce from 20 to the limiting value of 5 (assuming that the ICF volume is 0.5 ml per g - see Scharff and Wool, 1965a). In this connection it may be mentioned that similar findings have been reported in frog skeletal muscle. The data of Netter (1934) show an average T/P of 4.5 at higher external concentrations, rising to 9.6 at an external concentration of 160 μ mol/l, the lowest concentration tested. Conway and Moore (1945) also described an increase in T/P as the external concentration was decreased below 700 μ mol/l.

These observations are inconsistent with the view that ammonia is freely diffusible throughout the tissue, its distribution being controlled solely by its diffusion across the plasma membrane. The results suggest that the expected distribution is modified in some way. There could be permeability barriers within the tissue which limit the space accessible to the internal ammonia, or which prevent a portion of the tissue ammonia from exchanging with the extracellular

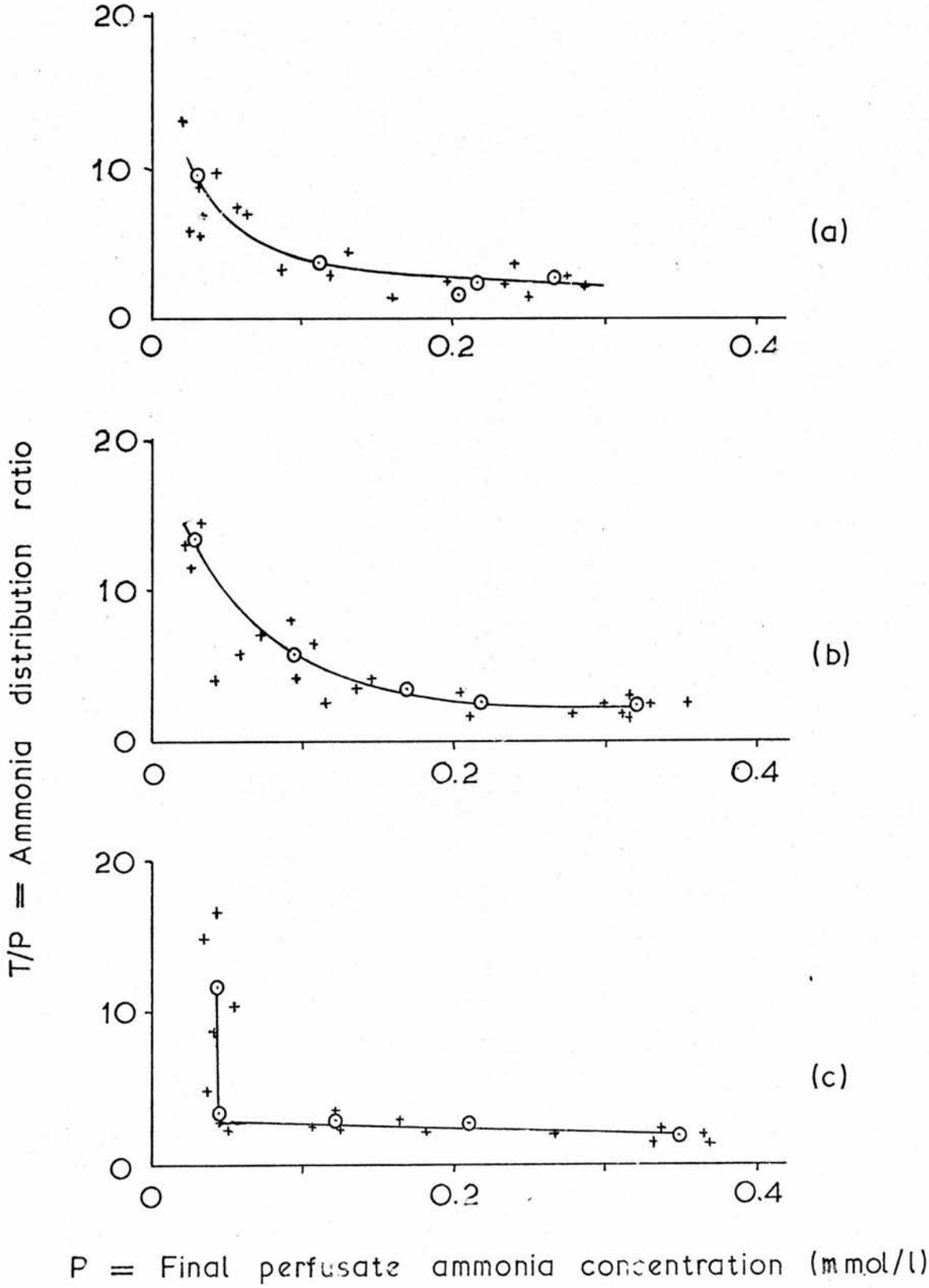
Fig. 3.11 The variation of the ammonia distribution ratio with the perfusate ammonia concentration.

The same data are used in this Figure as in Figs. 3.8 - 3.10, and are tabulated in Tables 3.6 - 3.3. The ratio of tissue ammonia to perfusate ammonia (T/P) is plotted against the perfusate ammonia concentration (P). The symbols are explained in the legend to Figs. 3.8 - 3.10.

(a) pH 7.39, K^+ normal

(b) pH 7.57, K^+ normal

(c) pH 7.39, K^+ 0.6 x normal.


20
10
0
0
0.2
0.4
(a)
(b)
(c)
T/P = Ammonia distribution ratio
P = Final perfusate ammonia concentration (mmol/l)

fluid. Or alternatively there may be no single process by which ammonia crosses the membrane, the mechanism at high external concentrations differing from that at low concentrations.

When the external ammonia concentration is low, the ratio Ci/Ce in the intact rat (Section 3.1), and that reported for frog muscle by Conway and Moore (1945), can approach the corresponding ratio for potassium. This suggests that in these circumstances the ammonia distribution is controlled by the membrane potential (Conway and Moore, 1945), which is itself effectively a function of the potassium distribution. Therefore ammonium ion would be the permeant species and would behave similarly to hydrogen ion (Sanslone and Muntwyler, 1965) and other monovalent ions such as rubidium and caesium (Hunter and Monahan, 1971). However, the fall in the distribution ratio as Ce is raised would suggest that some other process is involved at higher external concentrations. Rapid metabolic removal of tissue ammonia can be discounted since the net metabolic disappearance of ammonia is slow under all conditions (Section 3.2). Alteration of the membrane potential as the external concentration of ammonia rises can likewise be excluded, since the highest concentration involved is still very low in relation to the perfusate potassium concentration.

When no ammonium salt was added to the perfusate, reduction of the perfusate potassium by 40% increased the ratio T/P from 9.4 to 11.7 (Tables 3.6 and 3.8). However, this is not in itself a proof of dependence of the ratio on the membrane potential since at all other values of Ce, produced by addition of NH_4Cl, the effect of reduced external potassium was a reduction, or little change, in the ratio T/P. It is possible that the increase in T/P at the lowest

external concentration represents an effect of the decreased potassium on ammonia metabolism, since, unlike the hearts perfused with the standard medium, hearts perfused with reduced $[K^+]$ and low ammonia concentrations did not attain a constant perfusate ammonia concentration (Fig 3.2c). An excess of formation over disappearance within the tissue could result in a high T/P.

The slope of the graph of T against P (Fig 3.10) is perhaps a more reliable index of the way in which T varies with increasing P than is the absolute value of T/P at any single value of P. This slope is unaffected by the reduction in extracellular potassium provided that the extracellular ammonia concentration at the start of perfusion is greater than 60 μmol/l. At lower concentrations the sudden increase in T/P, already noted and possibly metabolic in origin, occurs. It seems unlikely therefore that diffusion of NH_4^+, controlled by the membrane potential, can account for the observed ammonia distribution.

It remains to consider diffusion of uncharged ammonia. In this case one would expect a value for the ratio Ci/Ce of 2.06 (Section 3.3.1.1), equivalent to a value of approximately 1.0 for T/P. This is certainly not the case when P is low, and even the limiting value of T/P when P is high never falls as low as this. In practice, T/P falls to a limiting value of around 2.5 on perfusion with standard medium (Fig 3.11). If the ammonia is distributed throughout the intracellular volume of 0.5 ml per g (Scharff and Wool, 1965a), it can be seen that Ci/Ce never falls below 5. Conversely, if all the internal ammonia is freely exchangeable with the external medium, a ratio of 2.5 for T/P would require an "intracellular volume" of 1.2 ml per g of tissue in order to maintain a true concentration

ratio (Ci/Ce) of 2.06. The internal concentration is thus too high to be solely the consequence of pH-dependent diffusion of NH_3.

3.4.2. Compartmentation of tissue ammonia

The anomalous dependence of T/P on P could be explained if it is assumed that ammonia is not freely diffusible throughout the tissue, but that a fraction of the tissue ammonia is prevented from exchanging with the perfusate ammonia. Under these circumstances a quantity of ammonia ("A" μ moles per g) would remain in the tissue even if the perfusate concentration were hypothetically reduced to zero (Fig 3.12). The diffusion of this fraction might be restricted either by intracellular permeability barriers (endoplasmic reticulum, for example) or by binding to structural elements of the tissue. This might be electrostatic attraction of NH_4^+ to some fixed anion, if the NH_4^+ cannot readily be exchanged for another cation. The remainder of the tissue ammonia ("mP" μ moles per g) would be free to exchange with the perfusate either as free ammonia or as ammonium ion. Tissue ammonia as measured (T) would be the sum of two intracellular pools, i.e. T = A + mP (Fig 12). Considering the distribution of ammonia between the exchangeable pool (mP) and the perfusate should then provide a clearer idea of the changes brought about by varying the pH or the potassium concentration of the perfusate than can be obtained by considering the tissue ammonia in toto.

In general terms, if the exchangeable pool occupies a space within the cell of v ml per g, its concentration (Ci') is mP/v μ moles per ml. If ammonium cation diffuses between the exchangeable pool and the ECF, reducing the external potassium concentration should bring

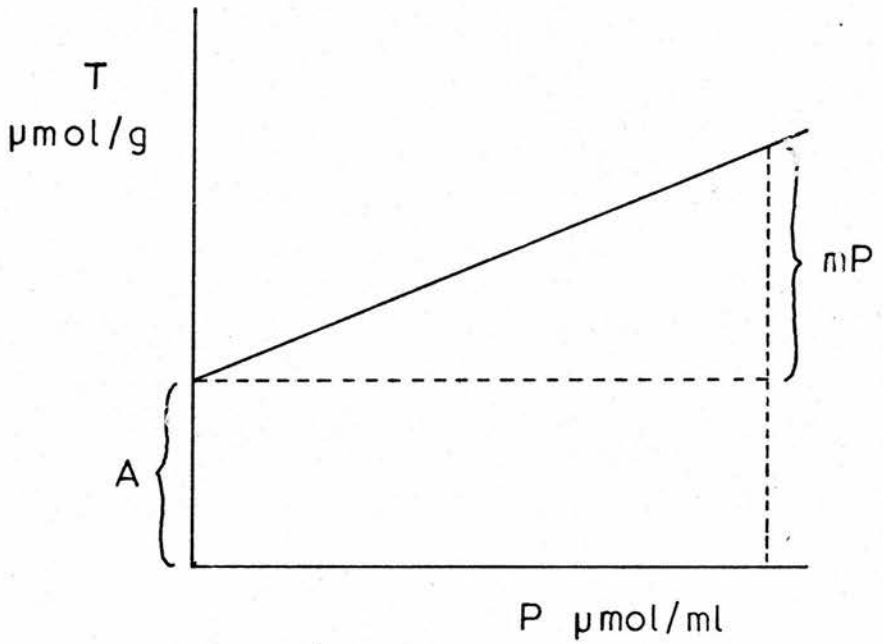


Fig. 3.12 Expected variation of tissue ammonia with perfusate ammonia: compartmental model.

The total tissue ammonia (T μmol/g) can be divided into two fractions: a non-exchangeable fraction (A μmol/g) which does not vary with perfusate ammonia concentration (P μmol/ml), and an exchangeable fraction, which does vary (mP μmol/g).

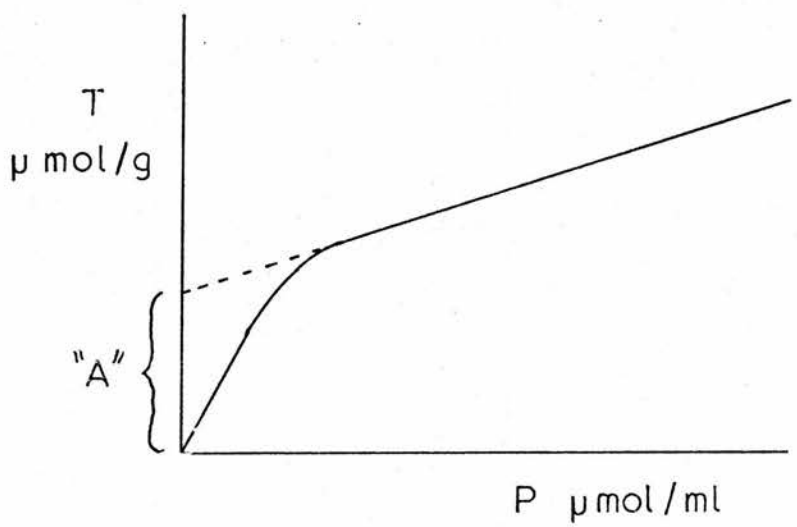


Fig. 3.13 Expected variation of tissue ammonia with perfusate ammonia: active transport of ammonia.

about an increase in Ci'/Ce (Section 3.3.1.2), hence, assuming that v remains constant, the slope (m) of the graph of T against P should increase. It has already been noted that this is not the case experimentally.

If uncharged ammonia diffuses, the distribution between the exchangeable pool and the ECF should behave as predicted above (Section 3.3.1.1) and Ci can be replaced by Ci' in equation (1):

$$\frac{Ci'}{Ce} = \frac{1 + 10^{(pKa - pHi)}}{1 + 10^{(pKa - pHe)}}$$

$$\text{Since } Ci' = \frac{mP}{v}, \text{ and } Ce = P$$

$$\frac{m}{v} = \frac{1 + 10^{(pKa - pHi)}}{1 + 10^{(pKa - pHe)}} \quad \ldots\ldots \quad (4)$$

Assuming that v is unaffected by altering the external pH, and that pHi, the pH of the space accessible to the exchangeable ammonia pool, is identical to the pHi measured by the DMO distribution, equation (4) predicts that an increase in pHe should bring about an increase in the slope (m) of the graph of T against P.

The observed slope of the graph at pH 7.57 is 1.02 (Section 3.3.2.2). The calculated value of the expression on the right-hand side of equation (4) at this pH is 3.09. Hence v = 0.33 ml per g. This is rather less than the volume of the intracellular space (Scharff and Wool, 1965a), but is not an unreasonable value. However, at pH 7.39 the fit is less good. The slope (m) is 1.71 and the calculated value of m/v is 2.06, giving a value for v of 0.85 ml per g,

similar to the volume of the total tissue water. Such a value is not possible if the internal ammonia is confined within the plasma membrane. Moreover, increasing pHe from 7.39 to 7.57 caused a decrease in (m) from 1.71 to 1.02, rather than the increase which is expected on theoretical grounds. These data would only fit the pH-dependent model if perfusion with the more alkaline medium caused a reduction in the volume accessible to the exchangeable pool. There is no a priori reason for supposing this to be true.

Although comparmentation into an exchangeable and a non-exchangeable pool cannot alone account for the observed ammonia distribution, the concept of compartmentation need not be rejected altogether at this stage. Experiments with labelled ammonia (Chapter 4) suggest that some form of compartmentation of tissue ammonia does indeed exist in heart muscle.

3.4.3 Active transport of ammonia

Specific ammonia-transporting systems have been described in some lower organisms (Hackette et al., 1970; Pribil and Kotyk, 1970); and transportation of ammonia by the Na^+/K^+-dependent ATPase of erythrocytes has also been demonstrated (Post and Jolly, 1957). It is not inconceivable therefore that such a process might occur in heart, which also has an Na^+/K^+-transporting ATPase which is capable of binding ammonia (Yoda and Hokin, 1972). An ammonia pump could be bi-directional or unidirectional. If ammonia substituted for potassium, as is the case with the erythrocyte ATPase (Post and Jolly, 1957), a unidirectional pump carrying ammonia into the tissue would result. This would tend to increase the distribution ratio Ci/Ce.

A carrier mechanism would display saturable kinetics. Let us assume that saturation is approached at the higher external concentrations used in these perfusion experiments (as is probably the case : see Chapter 4, Section 4.2.2), and that passive diffusion of ammonia also occurs. In this case the elevation of Ci/Ce would be greatest at low external concentrations. As Ce increased, the rate of passive influx would continue to increase proportionally whereas the rate of active transport would rise to a maximum as the carrier became saturated. Hence as Ce increased Ci/Ce would fall from an initial value, enhanced by the effect of active transport, to a limiting value characteristic of passive diffusion. The fall in Ci/Ce is in accordance with experimental findings although the limiting value of Ci/Ce does not closely correspond to diffusion either of NH_3 or of NH_4^+ as has already been shown (Section 3.4.1).

If the perfusate concentration were reduced to zero, the rate of uptake due to active transport would also be zero and the tissue concentration would depend on the rate at which ammonia was formed in the tissue. If this rate is low, then the tissue concentration could fall close to zero on account of the passive efflux of ammonia. As the external concentration approached zero therefore, the internal concentration would fall sharply (Fig 3.13). This is in contrast to the two-compartment system (Fig 3.12), in which a finite tissue concentration remained despite a perfusate concentration of zero. The perfusate system used (Chapter 2, Section 2.3.2.1) did not allow study of the heart at 75 minute perfusate concentrations less than 31 μmol/l, since ammonia produced by the heart during perfusion could not be removed from the system. Therefore the form of the lower part of the curve of Fig 3.13 could not be established by experiment. A

curve similar to that of Fig 3.13 was given by the ammonia distribution across the cell membrane in an alga in which the presence of an ammonia carrier was suspected (Pribil and Kotyk, 1970).

The effects of changes in pH or potassium concentration on such a carrier mechanism are difficult to predict. However, if potassium and ammonia compete for the transport system, reduction of the extracellular potassium should cause an increase in the ratio Ci/Ce for ammonia. Experimentally an increase was only observed at low initial Ce, as would be expected if the relative importance of the carrier became less as Ce increased. The possible role of metabolism in producing this increase has already been noted (Section 3.4.1).

3.4.4. The metabolism of ammonia by perfused rat heart

The formation or disappearance of ammonia (Section 3.2) is slow in comparison to other metabolic functions of the heart such as oxygen uptake or lactate production (Table 2.5), but is comparable in velocity to the amino acid changes described in Chapter 5. Alteration of the external pH had little effect on the metabolism of ammonia (Fig 3.1). There was no net metabolic utilization of ammonia in either case until the external concentration exceeded 200 μmol/l. This observation can be compared with that of Netter (1934) who showed that frog muscle did not take up ammonia from media in which its concentration was less than 710 μmol/l. Presumably the formation or disappearance of ammonia within the tissue is regulated by the tissue ammonia concentration. In the unperfused heart (Section 3.1) and in the heart *in vivo* (Stewart *et al.*, 1969) the internal ammonia concentration is approximately 0.4 μmoles per g fresh weight. It can be seen from the manner in which Ci varies with Ce on perfusion

(Tables 3.6 - 3.7; Figs 3.8 - 3.9) that the value of Ci does not greatly exceed the in vivo value until the initial Ce is 200 μmol/l or more. Hence there is a net disappearance of ammonia from the system only when Ci exceeds the "physiological" concentration, and the metabolic formation or disappearance of ammonia may be seen as a mechanism for keeping the ammonia concentration in the heart within defined limits. This mechanism is reinforced by the incomplete dependence of Ci on Ce shown by the ammonia distribution (Fig 3.8 - 3.9), which may be a result either of a non-exchangeable pool of ammonia in the tissue, acting as an "ammonia store", or a result of the active uptake of ammonia from media of low concentration by a "pump" mechanism.

The behaviour of hearts perfused with a medium of reduced potassium concentration was unexpected (Fig 3.1). Reduction of the initial external concentration from 330 μmol/l to 120 μmol/l had little effect on the net metabolism of ammonia, which on average did not differ significantly from zero over this range of concentrations. However, reduction of the initial Ce below 100 μmol/l resulted in an anomalous disappearance of ammonia from tissue and perfusate, seen in the fall in Ce during perfusion (Fig 3.5) and in the low internal concentration at the end of perfusion (Table 3.8; Fig 3.10). However, the heart does not appear to tolerate any further reduction in tissue concentration, since a further decrease in the initial Ce triggered a metabolic production of ammonia which restored Ci to the in vivo value (Table 3.8) and which was still continuing at the end of perfusion (Fig 3.2c).

3.4.5. Summary

The data presented in this chapter cannot be used to distinguish

conclusively between transport of NH_3 and NH_4^+, since neither hypothesis fits the data. Furthermore, the wide biological variation between hearts is bound to reduce the significance of any differences reported between values of quantities such as slopes and intercepts (Figs 3.8 - 3.10).

It is clear, however, that the involvement of ammonia in heart metabolism is much more complex than is usually imagined. The increase in the ratio Ci/Ce as Ce is reduced is in line with the earlier findings of Netter (1934) and Conway and Moore (1945) in frog muscle. This phenomenon may be a result of intracellular compartmentation of ammonia, or it may be a result of a different mechanism of transport becoming important at low external concentrations. The latter may involve an ammonia carrier.

In consequence the variation of Ci with Ce is less than would be the case if Ci were directly proportional to Ce. Similarly, metabolic synthesis or utilization of ammonia tends to restore the tissue ammonia concentration to the in vivo level. Hence a constant ammonia concentration in the intracellular fluid may be of more importance than is usually considered. The function of a constant ammonia concentration is a matter for speculation. Ammonia is an activator of phosphofructokinase (Abrahams and Younathan, 1971) and of certain other enzymes (see Chapter 1, Section 1.5). However, although a role in the activation of glycolysis in skeletal muscle is feasible, since in this muscle both ammonia production and glycolytic flux vary with activity (Chapter 1, Section 1.4), such a role in heart is unlikely since the glycolytic flux in this tissue is much more constant. Furthermore, the production of ammonia in heart does not appear to be linked to nucleotide deamination and

energy metabolism as it is in skeletal muscle (Chapter 5, Section 5.1). It is striking that concentrations of ammonia in the perfusate up to 0.3 m mol/l are not apparently toxic to the heart, despite the widely held belief that tissue ammonia reacts rapidly with α-oxo-glutarate, causing a depletion of the latter and a failure of energy metabolism (Chapter 1, Section 1.3). This may be responsible for the deleterious effects of ammonia on the brain (James et al., 1972). However, the fact that heart muscle tolerates relatively high intracellular ammonia concentrations without any decrease in oxygen consumption (Table 2.5) may mean that ammonia is prevented from reaching glutamate dehydrogenase, perhaps by permeability barriers or by the presence of cation binding sites which preferentially bind NH_4^+ and hinder its diffusion.

The net changes associated with the intracellular metabolism of ammonia and its transport across the plasma membrane have been measured and found to be small. It is the purpose of the following chapter to measure the absolute fluxes of ammonia in both directions across the membrane, to furnish further evidence for an ammonia transport system, and to investigate the possible intracellular compartmentation of ammonia.

CHAPTER 4

THE METABOLISM OF LABELLED AMMONIA IN PERFUSED RAT HEART

Chapter 4. The Metabolism of Labelled Ammonia in Perfused Rat Heart

Ammonia enriched with the stable isotope of nitrogen, ^{15}N, has been available for many years (Urey et al., 1937). Nevertheless, relatively little use has been made of it in the study of ammonia and amino acid metabolism in mammalian tissues. There has been a preference for working with amino acids labelled with the radioactive isotope of carbon, ^{14}C, since it is easier to measure than ^{15}N. It is not surprising therefore that a great deal more is known about the metabolism of the carbon skeletons of these compounds than about that of the amino group. In the present chapter a preliminary investigation into the uptake and metabolism of ^{15}N-labelled ammonia by rat heart is reported.

The use of ^{15}N in studies of nitrogen metabolism in mammalian systems began not long after enriched ammonia became available (Schoenheimer and Rittenberg, 1939). Methods similar to those used in the present study (Chapter 2, Section 2.3.6) were developed for the determination of isotope in organic nitrogen compounds and ammonia by conversion to nitrogen (Rittenberg et al., 1939), and the preparation of labelled amino acids was described (Schoenheimer and Ratner, 1939). The first metabolic studies involved the feeding of ^{15}N-ammonium citrate to rats (Foster et al., 1939; Schoenheimer, 1949). Analysis of amino acids in the carcass showed that the amide nitrogen fraction was the most highly labelled, followed by glutamate and aspartate. After injection of ^{15}N-labelled ammonia into rats (Duda and Handler, 1958) or rabbits (Ferdman et al., 1963), glutamine of high enrichment was found in all tissues, particularly in heart muscle. The time-course of labelling was not investigated, since in

both studies all animals were analyzed at a single time after injection. Thus the data do not necessarily indicate synthesis of labelled glutamine in the heart; glutamine synthesized in some distant organ such as the liver could pass into the heart from the plasma. (Uptake of glutamine by the perfused rat heart has been demonstrated in this study (Chapter 5, Section 5.2.5.1)). ^{15}N has also been used in studies on the incorporation of isotope from labelled ammonia into protein amide nitrogen (Vitti and Gaebler, 1963; Vitti et al., 1964) and in studies on the formation of ammonia from glutamine in the kidney (Pitts et al., 1965).

There is also a radioactive isotope of nitrogen, ^{13}N. Since this isotope has a half-life of only 10 minutes, it is best suited to experiments of short duration, or where a qualitative rather than a quantitative interpretation is desired (e.g. Monahan et al., 1972), and it can only be used when facilities for its preparation are at hand. Ammonia labelled with ^{13}N has been used in studies in live dogs. After injection, whole-body scanning showed that label became localized in heart, brain, liver, kidneys and bladder (Hunter and Monahan, 1971; Monahan et al., 1972). It was proposed that the heart took up NH_4^+ cation by a mechanism similar to the physiological uptake of potassium, or the uptake of rubidium or caesium used in myocardial studies (Hunter and Monahan, 1971). The possibility that the radioactivity found in the heart actually represented glutamine or some other labelled compound synthesized in another organ and taken up by the heart was discussed (Monahan et al., 1972), although the results of Harper et al. (1972) showed that the uptake of label by mouse heart was so rapid as to preclude this: nearly all the label which entered the heart did so on the first passage of the labelled

ammonia through the coronary circulation. 85% of the radioactivity of the blood disappeared from it in the first minute after injection: it was probable that the greater part of this represented uptake of ammonia by heart, lungs and possibly skeletal muscle (Bessman and Bessman, 1955). Owing to the short half-life of the isotope, its use in kinetic or metabolic studies is limited, and it is likely to find its widest application as a diagnostic tool in the visualization of myocardial disorders (Hoop et al., 1973).

4.1 Metabolite Changes after 15 Minutes Perfusion

Perfusion with labelled ammonia permits a study of the system in its approach to equilibrium. Therefore hearts must be perfused for a sufficient length of time to allow measurable changes to take place, yet not so long that the system reaches equilibrium. A perfusion time of 15 minutes was used in the present investigation. Hearts were perfused with the following media:

Serial No. of Hearts	Initial perfusate NH_4^+ concentration	Enrichment	Number of hearts perfused
104, 106	10 µmol/l	80 atoms % excess	2
107	160 µmol/l	31 atoms % excess	1
105, 108	360 µmol/l	19 atoms % excess	2

The number of hearts studied was limited by the availability of facilities for ^{15}N determination (See Chapter 2, Section 2.3.6).

Ammonia was measured in the perfusate at the beginning and end of perfusion, and in the tissue at the end of perfusion. Tissue glutamate was measured in one heart at each ammonia concentration.

Values for all hearts are shown in Table 4.1. Concentrations of ammonia and glutamate in the unperfused heart are taken from Chapter 5, Table 5.6.

The tissue glutamate concentrations after 15 minutes are similar to those measured at the end of 75 minutes, and demonstrate that the decrease in heart glutamate on perfusion (Chapter 5, Section 5.3.4) is complete after 15 minutes. During this period ammonia passes from the heart into media containing 10 µmol/l ammonia, whereas hearts perfused with the higher concentrations take it up. The high concentrations of ammonia in the tissue after 15 minutes deserve comment. The extent of ^{15}N-labelling of the tissue ammonia is very low in all hearts (Section 4.2 of this Chapter). Thus the excess ammonia does not derive from the medium, even in hearts perfused with media containing high concentrations of ammonia. It may be interpreted as an accumulation of ammonia in the tissue, indicating that the rate of its production during the first 15 minutes exceeds the rate at which it can diffuse out of the tissue. If this is correct, ½ to ¾ of the total ammonia (1.6 µmoles per g tissue in 75 minutes) produced by hearts perfused with media of low ammonia concentration is synthesized during the first 15 minutes of perfusion. Diffusion out of the heart is then a slower process, and net outward flux continues until a physiological tissue concentration is reached. The increase in total ammonia is therefore roughly parallel to the decrease in tissue glutamate. Alternatively, the high tissue ammonia may be due to an artifact; this is suggested particularly by the large variation, especially in the concentrations in the hearts perfused with high-ammonia media. Some component of the commercial ^{15}N-ammonium chloride preparation may have been poisoning the heart,

Metabolite	Heart Serial No.	Initial perfusate ammonia concentration, mmol/l	Tissue ammonia concentration, µmol/g		Change in concentration of metabolite in system, µmol/g		
			Unperfused	Perfused 15 min	Tissue	Perfusate	Total
Ammonia	104	0.010	0.43	0.89	+0.46	+0.36	+0.82
	106	0.010	0.43	1.35	+0.92	+0.40	+1.32
	107	0.160	0.43	1.00	+0.57	-3.12	-2.55
	105	0.360	0.43	2.25	+1.82	-3.01	-1.19
	108	0.360	0.43	0.54	+0.11	-4.26	-4.15
Glutamate	106	0.010	4.46	2.60	-1.86	-	-
	107	0.160	4.46	3.65	-0.81	-	-
	108	0.360	4.46	1.83	-2.63	-	-

Table 4.1 Metabolite changes after 15 minutes perfusion.

Ammonia was measured in tissue and perfusate by the enzymic method. Glutamate was measured in tissue by the hydroxylamine method (Chapter 2, Section 2.2.8.1). Concentrations in unperfused tissue are mean values (Chapter 5, Table 5.6).

Heart Serial No.	Initial perfusate ammonia concentration, mmol/l	Enrichment, Atoms % excess of ^{15}N				
		Perfusate			Tissue	
		Before perfusion	After perfusion	Approx. Dilution of isotope	Before perfusion	After perfusion
104	0.010	80.0	39.8	x2	0.0	0.78
106	0.010	79.2	40.5	x2	0.0	0.37
107	0.160	31.4	33.1	None	0.0	Not measured
105	0.360	18.3	19.1	None	0.0	1.5
108	0.360	19.1	19.1	None	0.0	10.3

Table 4.2 Changes in enrichment of tissue and perfusate ammonia on perfusion for 15 min with media containing ^{15}N-ammonium chloride.

Each heart was perfused with one of the three media of varying ammonia concentration described in the text. Ammonium ion was recovered from samples of perfusate before perfusion, and from perfusate and tissue after perfusion by the ion-exchange technique described in Chapter 2 (Section 2.3.6.1). The ^{15}N content was measured by mass spectrometry after conversion to nitrogen gas.

though the effect did not increase obviously with the concentration of ^{15}N-ammonia, and the ammonia content of the commercial material as measured by the routine enzymic assay (Chapter 2, Section 2.2.7) did not fall detectibly short of 100%. A further possibility is contamination of reagents by ammonia, though extracts of tissue from hearts 106-108 were made using fresh solutions of perchloric acid and potassium carbonate without causing the tissue ammonia to return to normal (except in heart 108).

4.2 The Labelling of Ammonia in Perfused Rat Heart - A Model System

Table 4.2 shows the effect of perfusion on the labelling of ammonia in perfusate and tissue. Isotope in the perfusate of hearts perfused with 10 µmol/l ammonia was diluted to half its initial concentration, whereas isotope in the perfusate of the other hearts was not detectibly diluted. Thus no detectible endogenous ammonia diffuses out of hearts perfused with 160 or 360 µmol/l ammonia in the first 15 minutes of perfusion. The enrichment of tissue ammonia is very low in comparison with that of perfusate ammonia in all hearts. The low enrichment of 1.5 for heart 105 is associated with a high tissue ammonia concentration (Table 4.1), and may reflect an abnormally large synthesis of unlabelled ammonia by this heart.

The data of Table 4.2 do not indicate how much isotope is taken up by the heart from the perfusate, or how much disappears in metabolism. However, the concentration of heavy ammonia in tissue and perfusate can be calculated if the total ammonia concentration and the isotope enrichment are known. Increases and decreases

in the isotope content of tissue and perfusate can be calculated from the data. Isotope concentrationsshown in Table 3 are μmoles $^{15}NH_4^+$ per g tissue in excess of the quantity of $^{15}NH_4^+$ (0.36%) present naturally in ammonia from all sources (Rittenberg et al., 1939).

Table 4.3 shows that $^{15}NH_4^+$ disappears from systems perfused for 15 minutes either with high or with low concentrations of ammonia. The disappearance represents metabolic utilization of ammonia. The most important feature of the data is that ^{15}N-ammonia is extensively metabolized by the heart without the tissue ammonia becoming labelled to any great extent. More than 25% of the isotope added to hearts 104 and 106 was metabolized by the tissue, although the enrichment of the tissue ammonia at the end of perfusion was only 0.01 to 0.02 times that of the perfusate. (If the enrichment of the ICF ammonia of heart 104 increased linearly from 0 to 0.78 atoms % excess during 15 minutes (Table 4.2), with an average value of 0.39, disappearance of 0.17 μmoles of $^{15}NH_4^+$ from a single pool of this size would represent metabolism of 0.17 x 100/0.39, or 43.6 μmoles of ammonia per g tissue, which is not feasible). The data lend support to the theory developed in Chapter 3 (Section 3.4.2), that two pools of tissue ammonia exist in heart. One pool is free to exchange with the perfusate, and it therefore becomes labelled by ammonia from the perfusate; the other pool is prevented from exchanging and remains unlabelled. The exchangeable pool alone is the source of the 0.17 μ moles of $^{15}NH_4^+$ per g tissue metabolized by heart 104.

Let A denote the non-exchangeable pool, and x the exchangeable pool; let X and Y represent undefined metabolites. A possible model is that of Fig 4.1, diagram 1. If it is assumed that the enrichment of the perfusate decreases linearly from 80 to 40 atoms % excess

	Initial perfusate ammonia concentration, mmol/l	0.010		0.160	0.360	
	Heart serial no.	104	106	107	105	108
Perfusate	Initial total ammonia	0.81	1.14	17.40	21.20	47.20
	Final total ammonia	1.16	1.54	14.28	18.18	42.70
	Initial ^{15}N-ammonia	0.64	0.90	5.75	3.87	9.00
	Final ^{15}N-ammonia	0.47	0.63	4.73	3.46	8.21
	Change in ^{15}N-ammonia	-0.18	-0.26	-1.02	-0.40	-0.79
Tissue	Final total ammonia	0.88	1.34	1.00	2.25	0.54
	Final ^{15}N-ammonia	0.007	0.005	Not measured	0.035	0.056
Whole system: Tissue + perfusate	Change in ^{15}N-ammonia	-0.17	-0.25	-	-0.36	-0.73
	Weight of heart, g	0.73	0.57	0.49	0.84	0.43

Table 4.3 Concentrations of total ammonia and isotope-labelled ammonia in tissue and perfusate.

The quantity of ammonia present in the perfusate before perfusion and in tissue and perfusate after 15 minutes perfusion is expressed as μmol/g heart. Total ammonia is that measured enzymically and is $^{14}NH_4^+ + {}^{15}NH_4^+$. The quantity of $^{15}NH_4^+$ present was calculated from this, knowing the enrichment (Table 4.2), and expressed as μmol/g heart.

The difference in the ammonia contents (as μmol/g) of the perfusates of hearts 105 and 108 reflect the disparity between the weights of the two hearts.

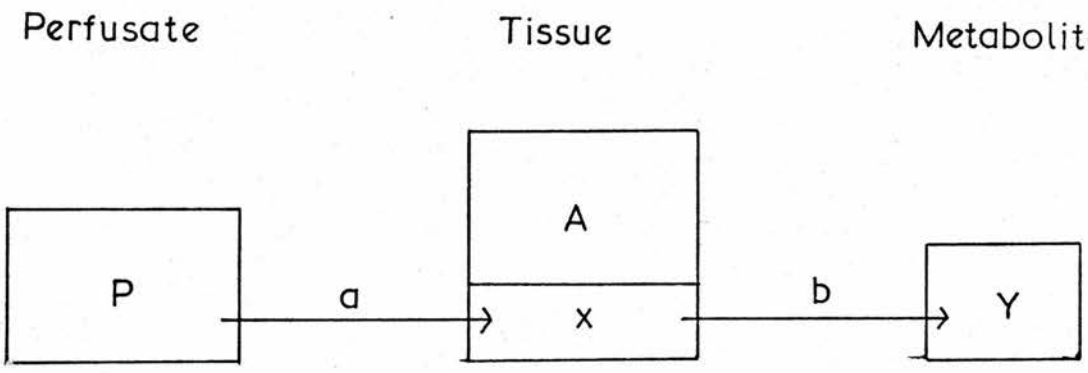


Diagram 1.

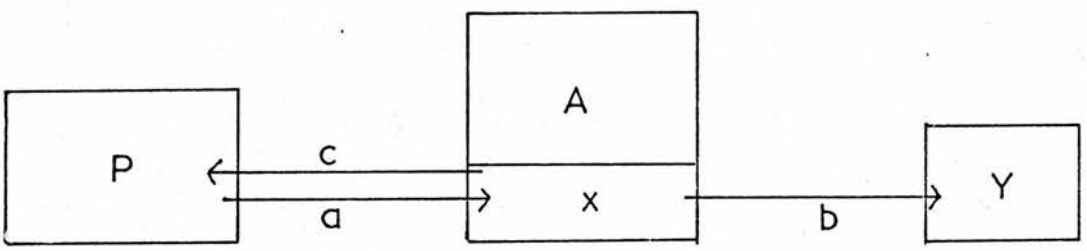


Diagram 2.

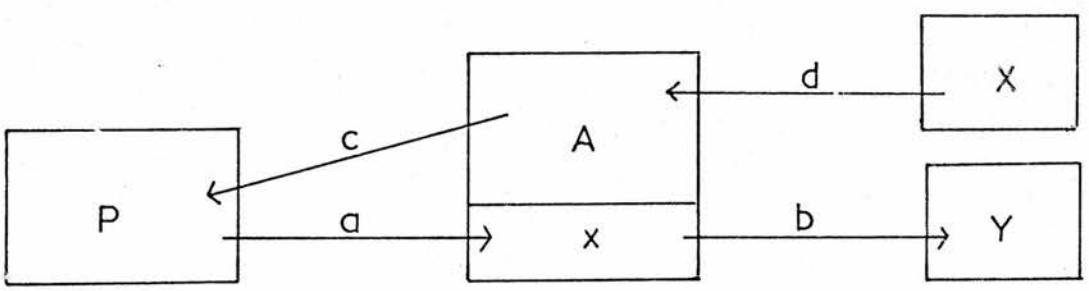


Diagram 3.

Fig. 4.1 Possible models of ammonia pools in perfused rat heart.

P = perfusate pool; A = non-exchangeable pool; x = exchangeable pool; X and Y = metabolites. Arrows (a) - (d) represent ammonia fluxes.

during 15 minutes, with an average value of 60, then the diffusion of 0.18 μ moles of $^{15}NH_4^+$ per g into the tissue (Table 4.3) is equivalent to a passage of 0.18 x 100/60, or 0.30 μ moles of ammonia per g into the heart. This is represented by arrow (a) in Fig 4.1. Since ammonia in the perfusate increases by 0.36 μ moles per g on perfusion (Table 4.1), there must be a balancing efflux of approximately 0.7 μ moles per g from the heart (arrow (c)).

In order to explain the observed dilution of label in the perfusate, this ammonia must be unlabelled and hence derive from the non-exchangeable pool (diagram (3) of Fig 4.1), rather than the exchangeable pool (diagram (2)), which ought rapidly to become labelled to the same enrichment as the perfusate. If it is assumed that the size of pool A is independent of the perfusate ammonia concentration, and that flux (c) depends on the size of pool A, then all hearts should release 0.7 μ moles of unlabelled ammonia per g into the perfusate in 15 minutes, irrespective of the ammonia concentration in the medium. In the case of heart 105, 18.18 μ moles of ammonia per g tissue are present in the perfusate at the end of perfusion (Table 4.3). The presence of 0.7 μ moles of unlabelled ammonia in this total would make little detectible difference to the enrichment of the perfusate ammonia. Hence no dilution of the isotope in the perfusate of this heart was detected experimentally. Pool A would require to be replenished from some metabolic source X, which may be glutamine (see Section 4.2.2). A simple model based on these experimental observations is depicted in Fig 4.1, diagram (3).

4.2.1 The parameters of the model

In the model depicted in Fig (4.1), diagram (3), fluxes (b)

and (d) are enzymic processes, whereas fluxes (a) and (c) represent transport of ammonia across the membrane. The rate of flux (b) is dependent on the size of the pool x; that of (c) on the size of pool (A); and that of (a) on the concentration of ammonia in the perfusate. The values of the parameters of the model (rate constants and the size of pool x) can be calculated for heart 104 if it is assumed that (c) is constant (for example if the size of A is constant throughout perfusion) and that (a) and (b) are directly proportional to the concentration of ammonia in the perfusate and in pool x respectively. This situation is shown in Fig 4.2, and the calculation (appendix 1) is based on this model. k_1 and k_2 are the rates of fluxes (a) and (b), λ_1 and λ_2 are the corresponding rate constants, and K is the constant rate of diffusion of ammonia from the non-exchangeable pool into the perfusate. Flux (d) is ignored in the calculation.

The parameters λ_1, λ_2 and K calculated from the data of heart 104 are shown in Table 4.4. If the same parameters are calculated from the data of heart 105 (a heart perfused with high ammonia medium), different values are obtained which are also shown in Table 4.4. The size of the exchangeable pool, and the concentration of ammonia in the perfusate after equilibrium is reached, have been calculated using the parameters and are likewise shown in Table 4.4. The calculated equilibrium concentration in the perfusate of heart 104 is exactly the same as that observed after 1 hour's perfusion.

The model thus provides an adequate explanation of the observations in hearts perfused with low ammonia, but not in hearts perfused with high ammonia concentrations. This is reflected by the negative values of K and of the equilibrium perfusate concentration and by the large value for the calculated size of the

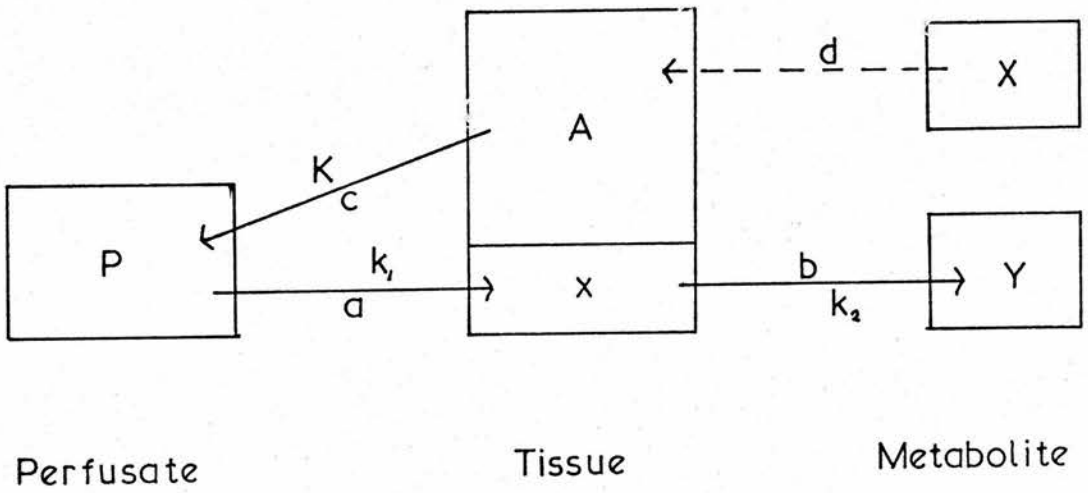


Fig. 4.2 The preliminary model.

k_1 and k_2 are the rates of ammonia fluxes (a) and (b), respectively; K is the constant rate of efflux of ammonia from the non-exchangeable pool into the perfusate. Other symbols are explained in Fig. 4.1.

	Heart 104		Heart 105	
	Calculated	Measured	Calculated	Units
$\lambda 1$	0.022	-	0.0074	$heart^{-1}.min^{-1}$
$\lambda 2$	1.54	-	0.75	$heart^{-1}.min^{-1}$
K	0.033	-	-0.0465	$\mu mol.heart^{-1}.min^{-1}$
$[S_x]_{15}$	0.016	-	1.02	$\mu mol.g^{-1}$
Perfusate ammonia Concentration at equilibrium	0.030	0.031	-6.28	$\mu mol.ml^{-1}$

Table 4.4 Parameters of the first model (see Fig 4.2).

λ_1 and λ_2 are the respective rate constants for diffusion of ammonia into, and out of, the exchangeable pool. K is the constant rate of diffusion of ammonia from the non-exchangeable pool into the perfusate. $[S_x]_{15}$ is the quantity of ammonia in the exchangeable pool after 15 minutes perfusion. The measured perfusate ammonia concentration at equilibrium is the mean of 9 determinations in hearts perfused for 75 min with media of average initial ammonia concentration 0.007 mmol/l. A specimen calculation of the parameters of heart 104 is given in appendix 4 I.

exchangeable pool: the model predicts that more ammonia enters the heart than is observed experimentally. If the rate of influx of ammonia (k_1) from the perfusate to the exchangeable pool were hyperbolically, rather than linearly, related to the perfusate concentration (e.g. $k_1 = \frac{V.Sp}{K' + Sp}$, where V and K' are constants, and S_p is the perfusate ammonia concentration, this error would be reduced. Such would be the case if ammonia uptake were mediated by a saturable carrier mechanism. Furthermore, the rate of efflux from the non-exchangeable pool (A) to the perfusate may not be constant if the size of A varies during perfusion. These possibilities are considered below.

4.2.2 The Optimum Model

In the model depicted in Fig 4.3, fluxes (b) and (c) are proportional to the size of pools A and x respectively; (a) is a hyperbolic function and is saturated at high perfusate ammonia concentrations; and (d) represents the enzymic breakdown of a precursor. Since glutamine was the only amino acid tested which significantly increased myocardial ammonia production (Chapter 5, Section 5.2.5.1), flux (d) has been taken to represent the action of glutaminase on the large pool of glutamine in the heart; the kinetic constants are those measured by Ottaway (1969b) for rat heart glutaminase. Flux (b) is also an enzymic reaction; if, however, the concentration of ammonia in pool (x) is low relative to the Michaelis constant of the enzyme, as would presumably be the case if this were glutamate dehydrogenase with a Km of 3.2 m mol/l (Frieden, 1963), the relation of (b) to S_x can be assumed to be linear.

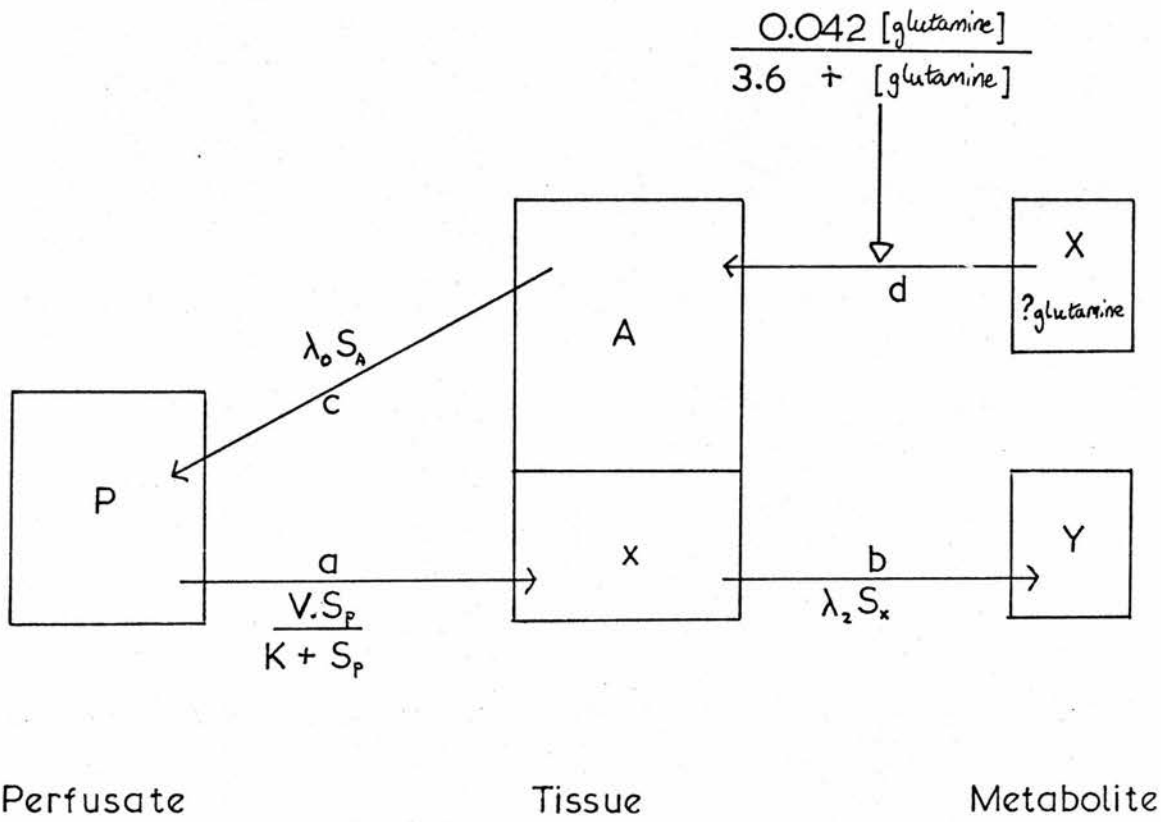


Fig. 4.3 The optimum model of the behaviour of ammonia in perfused rat heart.

S_A, S_x and Sp denote ammonia concentrations in tissue pools A and x, and in the perfusate, respectively. λ_0 and λ_2 are rate constants for fluxes (c) and (b). Flux (a) is a saturable process with kinetic constants K and V. Flux (d) is assumed to represent the hydrolysis of glutamine with constants as measured by Ottaway (1969b) for rat heart glutaminase.

By computer, values of the parameters λ_o, V, K, λ_2 and $(Sx)_o$ can be chosen so as to produce the minimum deviation of the calculated pool sizes from those measured experimentally in the five hearts studied. The chosen values of the parameters are shown in Table 4.5, and the calculated pool sizes at 0 and 15 minutes are compared with those measured experimentally in Table 4.6. Calculated pool sizes at 75 minutes are shown in Table 4.7 and compared with those measured in hearts perfused for 75 minutes.

The fit of the model to the data is very good except for the case of the tissue ammonia in hearts perfused with 360 μmol/l ammonia, where the two hearts show wide variation. If the model is correct, the size of the non-exchangeable pool (A) does not remain constant, but increases at least during the first 15 minutes of perfusion. The value of K = 4.16 μmol/heart (Table 4.5) for the uptake of ammonia from the perfusate is equivalent to a perfusate ammonia concentration of 83.2 μmol/l. Thus one would expect the carrier mechanism to become saturated at the higher perfusate ammonia concentrations (e.g. 310 μmol/l) used in the experiments described in Chapter 3. This could account for the observed variation of tissue ammonia concentration with perfusate ammonia concentration (see Chapter 3, Section 3.4.3). Calculation of pool sizes at 75 minutes (Table 4.7) shows that the calculated perfusate concentration reached in hearts perfused with 10 μmol/l ammonia (31 μmol/l) is identical to that measured experimentally, although the predicted 75 minute concentration in the perfusate of hearts perfused with 160 or 360 μmol/l ammonia is lower than that observed experimentally. Furthermore, the calculated amounts of ammonia in the tissue of hearts perfused with all media are greater than those reported in Chapter 3, Section 3.3.2.1.

V	2.31	$\mu mol.heart^{-1}.min^{-1}$
K	4.16	$\mu mol.heart^{-1}$
λ_o	0.040	$heart^{-1}.min^{-1}$
λ_2	1.41	$heart^{-1}.min^{-1}$
$[S_x]_o$	0.0031	$\mu mol.heart^{-1}$

Table 4.5 Parameters of the optimum model.

The optimum model is depicted in figure 4.3, where the meaning of V, K, λ_o and λ_2 is explained. $[S_x]_o$ is the quantity of ammonia in the exchangeable pool at the start of perfusion. These five quantities were chosen by computer so as to produce the minimum deviation of calculated pool sizes from those measured experimentally in the five hearts studied.

Legend to Table 4.6

The sizes of all ammonia pools are shown as μmol ammonia/heart. S refers to total ammonia ($^{14}NH_4^+ + {}^{15}NH_4^+$). R refers to $^{15}NH_4^+$. Subscripts p, x and A denote the ammonia pools : perfusate, exchangeable and non-exchangeable, respectively. Subscripts 0 and 15 denote the time of perfusion (min). Hence $[R_p]_0$ is the initial $^{15}NH_4^+$ content of the perfusate as μmol/heart, $[S_A + S_x]_{15}$ is the total ammonia in both tissue pools after 15 min perfusion, etc.

$[S_A + S_x]_0$, the tissue ammonia before perfusion, is calculated from the average value of 0.43 μmol/g in unperfused hearts (Chapter 5, Table 5.6). Thus for a heart weighing an average 0.6 g, $[S_A + S_x]_0 = 0.43 \times 0.6 = 0.26$ μmol/heart. $[S_x]_0$ is the value chosen by the computer as given in Table 4.5. Calculated values for other initial pool sizes are the average of measured results.

Using the above values for zero time pool sizes. 15 minute pool sizes were calculated by computer using parameters (Table 4.5) chosen to give the best fit. Table 4.6 shows the comparison between these values and the values obtained by measurement after 15 minutes perfusion.

Initial perfusate ammonia concentration	0.010 mmol/l			0.160 mmol/l		0.360 mmol/l		
	Measured		Calculated	Measured	Calculated	Measured		Calculated
Heart serial no.	104	106		107		105	108	
$[S_p]_o$	0.59	0.65	0.62	8.52	8.52	17.80	20.30	19.05
$[S_p]_{15}$	0.85	0.88	0.75	6.99	7.28	15.27	18.47	16.11
$[R_p]_o$	0.47	0.51	0.49	2.82	2.82	3.25	3.87	3.56
$[R_p]_{15}$	0.34	0.36	0.40	2.32	2.33	2.91	3.53	2.97
$[S_x]_o$	-	-	0.003	-	0.003	-	-	0.003
$[S_x]_{15}$	-	-	0.007	-	0.006	-	-	0.141
$[S_A + S_x]_o$	0.26	0.26	0.26	0.26	0.26	0.26	0.26	0.26
$[S_A + S_x]_{15}$	0.64	0.78	0.57	0.49	0.63	1.89	0.23	0.70
$[R_x]_o$	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
$[R_x]_{15}$	0.005	0.003	0.004	Not measured	0.021	0.029	0.024	0.026

Table 4.6 Pool sizes measured in hearts after 15 minutes perfusion, compared with those calculated from the model of Fig 4.3 as described in the text.

Initial perfusate Ammonia concentration, mmol/l	0.007	0.010	0.205	0.160	0.310	0.360
Size of pool	Measured	Calculated	Measured	Calculated	Measured	Calculated
$[S_P]_{75}$, µmol.heart^{-1}	-	1.53	-	4.58	-	8.84
P_{75}, mmol/l	0.031	0.031	0.216	0.092	0.266	0.177
$[S_x]_{75}$, µmol.heart^{-1}	-	0.014	-	0.042	-	0.080
$[S_A + S_x]_{75}$, "	0.19	0.72	0.31	0.75	0.46	0.78

Table 4.7 Pool sizes after 75 minutes perfusion.

P_{75} denotes the concentration of ammonia in the perfusate after 75 min. Other symbols are explained in the legend to Table 4.6. 75 min pool sizes predicted by computer are compared with those measured in tissue and perfusate of hearts perfused for 75 min with media nearest in ammonia concentration to those used in this chapter. $[S_A + S_X]_{75}$, the tissue ammonia content after 75 min, was measured in perchlorate extracts of the same hearts; the values given have been corrected for heart weight assuming an average weight of 0.6 g.

It is possible that the picture is distorted by the high 15 minute tissue ammonias of hearts 104-108, on which the calculation is based; these may after all be due to an artefact, and perhaps should not be relied upon too heavily.

4.3 The Metabolic Fate of Ammonia

Although hearts perfused with 300 μmol/l ammonia metabolize 3.5 μmoles of ammonia per g heart during 75 minutes (Chapter 3, Section 3.2.1), no significant increase in synthesis of any amino acid by the heart was observed (Chapter 5, Section 5.2.4). There are two reactions available in peripheral tissues for the fixation of ammonia. Glutamine synthetase may not occur in rat heart (Iqbal and Ottaway, 1970; see Chapter 1, Section 1.2). Glutamate dehydrogenase on the other hand is more active in heart than it is in muscle (Lowenstein, 1972). If the primary event in the fixation of ammonia is the synthesis of glutamate, it should be possible to detect labelled glutamate in the tissue.

However, if isotope is measured in tissue glutamate (Chapter 2, Section 2.3.6.3), it is found that the enrichment is very low; in Table 4.8 the measured ^{15}N-glutamate is compared with the value calculated by computer using the model of Section 4.2.2. (Fig 4.3), assuming that glutamate is the first product formed from the ammonia in the exchangeable pool. Thus the label disappearing from ammonia is not found in glutamate, and there are two possible explanations for this phenomenon. The first product formed from ammonia may not in fact be glutamate. If it is glutamine, glutamine labelled in the amide nitrogen should be measurable by appropriate techniques, and

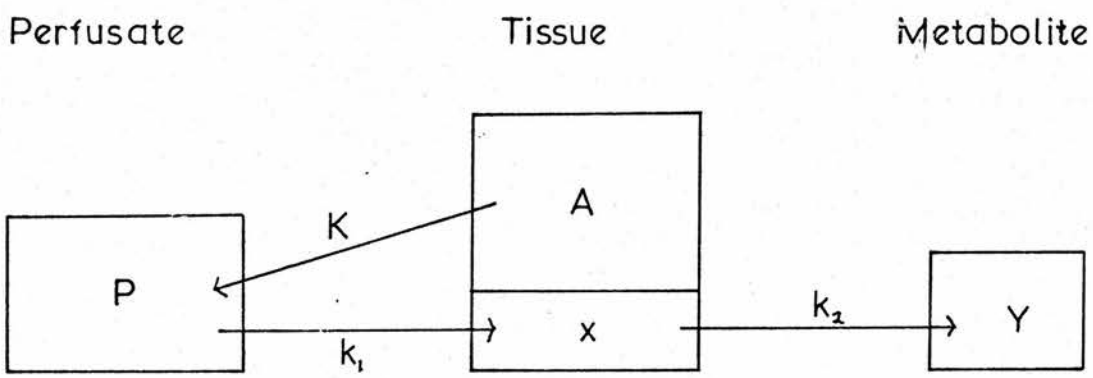
Initial perfusate Ammonia concentration	0.010 mmol/l		0.160 mmol/l		0.360 mmol/l	
Heart serial no.	106		107		108	
	Measured	Calculated	Measured	Calculated	Measured	Calculated
Total glutamate, μmol.heart^{-1}	1.48	-	1.79	-	0.79	-
^{15}N-glutamate "	0.010	0.079	0.020	0.291	0.002	0.228

Table 4.8 Labelling of glutamate in perfused rat heart.

^{15}N-glutamate was measured in perchloric extracts of tissue after perfusion for 15 minutes as described in the text. The measured value is compared with that calculated by computer, using the model of Fig 4.3 and assuming that glutamate is the first product formed from the ammonia in the exchangeable pool. Values are expressed as μmol/heart.

such a finding would demonstrate the existence of glutamine synthetase in rat heart. Alternatively, glutamate may be formed first, but may rapidly transaminate to yield labelled aspartate or alanine, which should also be measurable. If the second explanation is correct it must be assumed that ^{15}N enters and leaves heart glutamate without the latter becoming highly labelled, as is the case with heart ammonia. Hence tissue glutamate may also exist in two pools, only one of which is in equilibrium with the exchangeable ammonia pool and is labelled from it. It is likely that the physical location of the labelled glutamate pool is the same as that of the labelled ammonia pool. This suggests a compartment concerned with the active metabolism of several interrelated substances. The possible location of this compartment is discussed in Chapter 6 on the basis of histochemical studies. Such an arrangement would have the further advantage of allowing a small portion of the tissue ammonia to react with glutamate dehydrogenase, whilst the larger fraction (pool A) is prevented from doing so, thereby preventing depletion of tissue α-oxoglutarate and failure of energy metabolism, which occurs in brain (James et al., 1972).

Appendix 4.I Calculation of model parameters and pool sizes.



k_1, k_2 and K are the rates of the ammonia fluxes shown (as μmol.heart^{-1}.min^{-1}). K is a constant rate. λ_1 and λ_2 are the rate constants corresponding to k_1 and k_2.

Let isotope in perfusate = R_P μmol/heart.

Let enrichment = a_P ($\equiv 100a_P$ atoms % excess).

Let total ammonia in perfusate = S_P μmol/heart.

Hence $R_P/S_P = a_P$.

Similarly, let isotope in pool x = R_x;

isotope in pool A = R_A; etc.

Consider isotope in perfusate pool P:

Rate of increase of R_P

$$= \frac{dR_P}{dt} = -k_1 . a_P = -\frac{k_1}{S_P} . R_P .$$

Rearranging, $$\frac{dR_P}{R_P} = -\frac{k_1}{S_P} . dt.$$

Integrating, $$\log_e R_P = -\frac{k_1}{S_P} t + c.$$

When $t = 0$, let $R_P = [R_P]_o$.

Hence $c = \log_e [R_P]_o$.

$$\therefore \log_e R_P = -\frac{k_1}{S_P} t + \log_e [R_P]_o$$

$$\therefore \log_e \frac{R_P}{[R_P]_o} = -\frac{k_1}{S_P} t \quad \ldots\ldots\ldots\ldots (1)$$

or $$R_P = [R_P]_o . e^{-(k_1/S_P)t}.$$

From (1), $$\log_e \frac{[R_P]_o}{R_P} = \frac{k_1}{S_P} t$$

But since $k_1/S_P = \lambda_1$, $$\log_e \frac{[R_P]_o}{R_P} = \lambda_1 t \quad \ldots\ldots\ldots (2)$$

Consider heart no. 104:

$[R_P]_o = 0.47$; $[R_P]_{15} = 0.34$; $t = 15$.

Substituting in (2), $\lambda_1 = 0.0216 \text{ heart}^{-1} . \text{min}^{-1} = 0.0296 \text{ g}^{-1} . \text{min}^{-1}$.

Consider total ammonia in perfusate pool:

Rate of increase of S_P

$$= \frac{dS_P}{dt} = -k_1 + K.$$

But $k_1 = \lambda_1 S_P$; K is constant.

$$\frac{dS_P}{dt} = -\lambda_1 S_P + K.$$

Integrating,

$$S_P.e^{\lambda_1 t} = K\int e^{\lambda_1 t}.dt = \frac{K}{\lambda_1}.e^{\lambda_1 t} + c.$$

When $t = 0$, $S_P = [S_P]_o$.

$$\therefore c = [S_P]_o - \frac{K}{\lambda_1}.$$

$$\therefore S_P.e^{\lambda_1 t} = \frac{K}{\lambda_1}e^{\lambda_1 t} + [S_P]_o - \frac{K}{\lambda_1}$$

$$\therefore S_P = \frac{K}{\lambda_1}(1 - e^{-\lambda_1 t}) + [S_P]_o.e^{-\lambda_1 t} \quad \ldots\ldots\ldots\ldots(3)$$

In heart no. 104 at $t = 15$,

$$S_P = 0.95; \quad \lambda_1 = 0.0216; \quad [S_P]_o = 0.59.$$

Substituting in (3), $K = 0.033\ \mu mol.heart^{-1}.min^{-1}$.

When equilibrium is reached,

$$K = k_1 = \lambda_1 S_P.$$

$$\therefore S_P = K/\lambda_1 = 0.033/0.0216 = 1.53\ \mu \text{ moles.}$$

Thus in a perfusate volume of 50 ml,

Concentration of ammonia in perfusate at equilibrium

$$= 1.53/50 \times 10^3 = 31\ \mu mol/l.$$

Consider isotope in exchangeable pool (x) of heart:

Rate of increase of R_x

$$= \frac{dR_x}{dt} = k_1 a_P - k_2 a_x$$

$$= \frac{k_1}{S_P} \cdot R_P - \frac{k_2}{S_x} \cdot R_x$$

$$= \lambda_1 R_P - \lambda_2 R_x.$$

Since $R_P = [R_P]_o \cdot e^{-\lambda_1 t}$ (see (1) above),

$$\frac{dR_x}{dt} + \lambda_2 R_x = \lambda_1 [R_P]_o \cdot e^{-\lambda_1 t}.$$

Integrating,

$$R_x \cdot e^{\lambda_2 t} = \lambda_1 [R_P]_o \int e^{(\lambda_2 - \lambda_1)t} \cdot dt$$

$$= \frac{\lambda_1}{\lambda_2 - \lambda_1} [R_P]_o \cdot e^{(\lambda_2 - \lambda_1)t} + c.$$

When $t = 0$, $R_x = 0$, hence $c = -\frac{\lambda_1}{\lambda_2 - \lambda_1} [R_P]_o$.

$$R_x e^{\lambda_2 t} = \frac{\lambda_1}{\lambda_2 - \lambda_1} [R_P]_o \left\{ e^{(\lambda_2 - \lambda_1)t} - 1 \right\}$$

$$R_x = \frac{\lambda_1}{\lambda_2 - \lambda_1} [R_P]_o \cdot (e^{-\lambda_1 t} - e^{-\lambda_2 t}) \quad \ldots\ldots\ldots (4).$$

In heart no. 104, when $t = 15$, $R_x = 0.005$. $\lambda_1 = 0.0216$; $[R_P]_o = 0.47$.

Substituting in (4), $\underline{\lambda_2 = 1.54 \text{ heart}^{-1} \cdot \text{min}^{-1}}$.

Consider total ammonia in exchangeable pool:

Rate of increase of S_x

$$= \frac{dS_x}{dt} = k_1 - k_2$$

$$= \lambda_1 S_P - \lambda_2 S_x.$$

Integrating,

$$S_x.e^{\lambda_2 t} = \frac{\lambda_1 K}{\lambda_2} \int e^{\lambda_2 t}.dt + \lambda_1([S_P]_o - \frac{K}{\lambda_1}).\int e^{(\lambda_2-\lambda_1)t}.dt$$

$$= \frac{K}{\lambda_2}.e^{\lambda_2 t} + \left\{\frac{\lambda_1[S_P]_o - K}{\lambda_2 - \lambda_1}\right\}.e^{(\lambda_2-\lambda_1)t} + c.$$

When $t = 0$, $S_x = [S_x]_o$.

$$\therefore c = [S_x]_o - \frac{K}{\lambda_2} - \frac{\lambda_1[S_P]_o - K}{\lambda_2 - \lambda_1}.$$

$$\therefore S_x = [S_x]_o.e^{-\lambda_2 t} + \frac{K}{\lambda_2}(1 - e^{-\lambda_2 t}) + \frac{\lambda_1[S_P]_o - K}{\lambda_2 - \lambda_1}(e^{-\lambda_1 t} - e^{-\lambda_2 t}) \quad .....(5)$$

If $e^{-\lambda_2 t}$ is very small ($= e^{-23.1}$ in heart 104),

$$S_x \simeq \frac{K}{\lambda_2} + \frac{\lambda_1[S_P]_o - K}{\lambda_2 - \lambda_1}(e^{-\lambda_1 t}) \quad .....(6)$$

In heart 104, $\lambda_1 = 0.0216$; $\lambda_2 = 1.54$; $K = 0.033$; $[S_P]_o = 0.59$.

Substituting in (6),

$$S_x = \underline{0.012\ \mu mol.heart^{-1}.}$$

Since heart no. 104 weighed 0.73g, $S_x = 0.016\ \mu mol.g^{-1}$.

Since the total tissue ammonia at 15 minutes is $0.64\ \mu mol.heart^{-1}$,

S_x is 2% of the total tissue ammonia.

CHAPTER 5

AMMONIA AND AMINO ACID METABOLISM IN PERFUSED RAT HEART

Chapter 5 Ammonia and Amino Acid Metabolism in Perfused Rat Heart

It has been shown (Chapter 3, Table 3.4) that perfused rat heart produces substantial quantities of ammonia, 1.5 μ moles per g fresh weight in the first 60 minutes of perfusion. Keul and his group (1964, 1966) have shown that amino acids do not contribute significantly to the energy metabolism of the human heart *in vivo*. However, results discussed in the latter half of this chapter show the extent to which their oxidation does occur in the isolated perfused rat heart (see also Buse *et al.*, 1972). Deamination of amino acids results in the production of ammonia. In this chapter three possible mechanisms of ammonia production are discussed. Firstly, catalytic deamination of aspartate, for example by the purine nucleotide cycle (Lowenstein, 1972); secondly, direct deamination of glutamate by glutamate dehydrogenase; thirdly, hydrolysis of glutamine (Ottaway, 1969a,b). The overall result would be similar in the first two cases, but the direct precursor of ammonia in the first case would be an adenine nucleotide (or nucleoside), and in the second case, an amino acid.

5.1 Adenine Nucleotides and Ammonia

Anoxia in skeletal muscle leads to the production of large quantities of ammonia (Parnas and Mozolowsky, 1927). This is due to the accummulation of AMP, which is hydrolyzed to IMP and ammonia (Schmidt, 1928). Under physiological, aerobic conditions a pathway operates which reconverts IMP to AMP (Newton and Perry, 1960; Davey, 1961). The pathway requires energy in the form of GTP. These reactions form a cycle in skeletal muscle, the net result of which is the

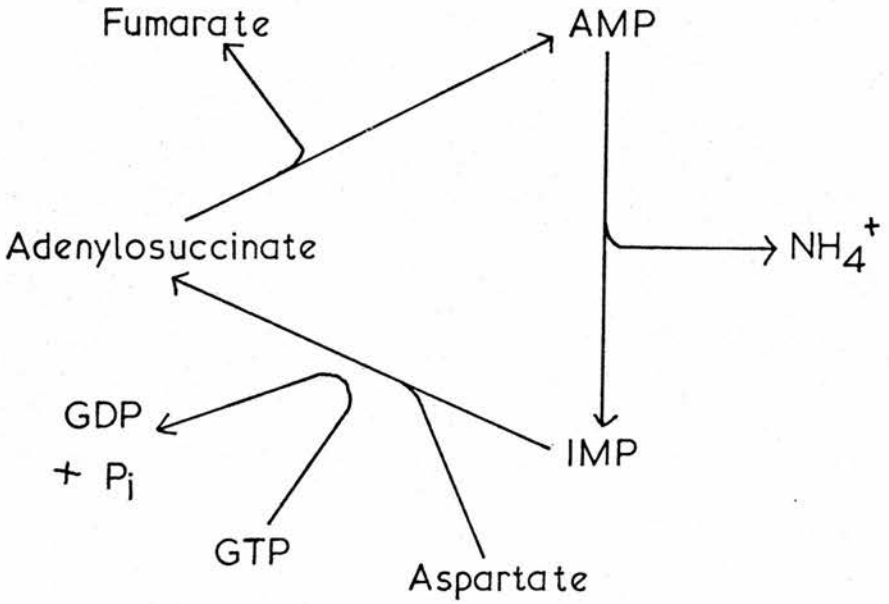


Figure 5.1. The purine nucleotide cycle.

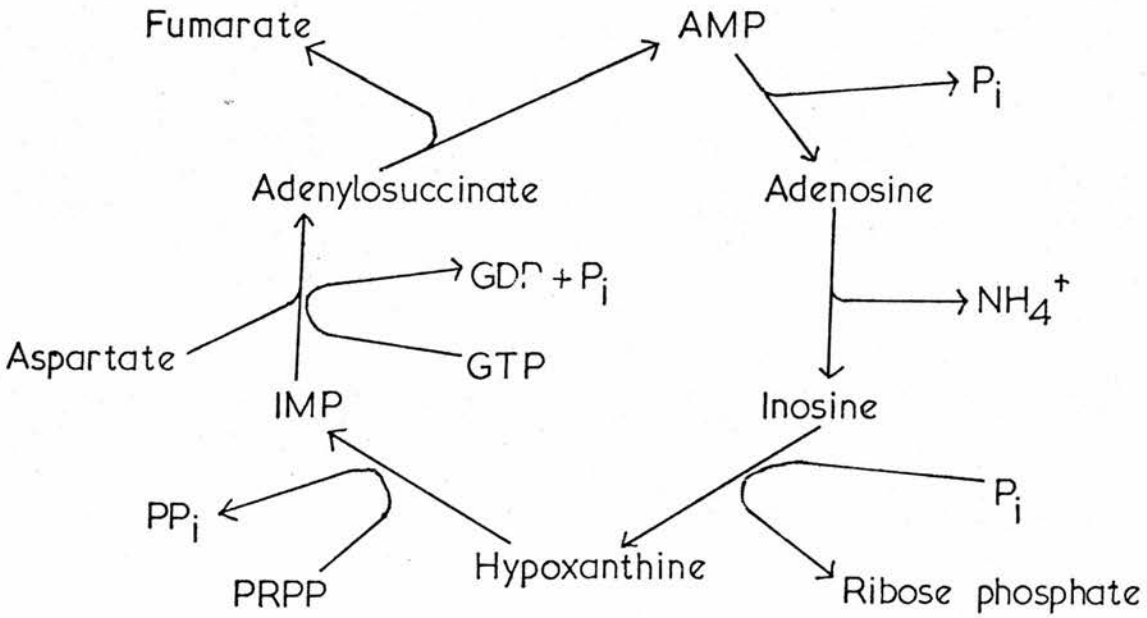


Figure 5.2. The purine nucleoside cycle.

P_i = inorganic phosphate; PP_i = inorganic pyrophosphate;
PRPP = 5-phosphoribosylpyrophosphate.

	Concentration in tissue (μmol/g fresh weight)						
	Control Ventricle	Ventricle after ischaemia for the times shown (min)					
		5	10	20	30	45	60
ATP	4.28	3.39	2.70	0.46	0.24	0.14	0.08
ADP	1.05	1.75	1.78	0.90	0.85	0.70	0.45
AMP	0.16	0.42	0.69	2.74	2.67	2.38	2.25
Adenosine	0.00	0.02	0.13	0.50	0.48	0.32	0.25
IMP	0.00	0.03	0.03	0.08	0.13	0.22	0.28
Inosine	0.00	0.02	0.20	0.74	0.86	1.37	1.61
Adenine	0.00	0.00	0.02	0.03	0.08	0.05	0.04
Hypoxanthine	0.00	0.01	0.07	0.18	0.24	0.37	0.46
Total	5.49	5.64	5.62	5.63	5.55	5.55	5.42

Table 5.1 Concentrations of adenine and hypoxathine nucleotides in rat heart during anoxia (reproduced from Gerlach et al., 1963).

The data show that much of the ATP and ADP which disappears during anoxia is converted to AMP and inosine. There is a transient peak in the concentration of adenosine, suggesting that this, rather than IMP, is the intermediate in the conversion of AMP to inosine. The concentration of IMP does not increase significantly until towards the end of the experiment.

hydrolysis of aspartate to fumarate and ammonia (Fig 5.1). Thus one of the suggested functions of the cycle (Lowenstein, 1972) is the deamination of amino acids which can donate their amino-group, via glutamate, to aspartate.

In hypoxic cardiac muscle on the other hand, deamination of AMP is much less important than deamination of adenosine to inosine, as is shown by the data of Gerlach et al. (1963), reproduced in Table 5.1, and confirmed by the data of Richman and Wyborny (1964). This is due to two factors: firstly, the high activity of 5′-nucleotidase of heart muscle (Baer et al., 1966), which competes more successfully for AMP than does AMP deaminase; and secondly, the presence of cytoplasmic adenosine deaminase in this tissue. AMP deaminase in heart is less active and chiefly found in the nucleus (Baer et al., 1966), where it may be concerned in nucleic acid metabolism.

Under aerobic conditions inosine can be recoverted to adenine nucleotides (Tsuboi and Buckley, 1965; Liu and Feinberg, 1971). The pathway begins with conversion of inosine to hypoxanthine. The key enzymes, purine nucleoside phosphorylase and hypoxanthine phosphoribosyltransferase, are present in heart (Maguire et al., 1972). Again one can postulate a cyclic pathway in aerobic tissue (Fig 5.2). Like the Lowenstein cycle, this cycle results in the deamination of aspartate. It is, however, a very expensive process energetically, and its primary functions are probably connected with salvage of purine nucleotides (Maguire et al., 1972) and regulation of the concentration of adenosine, a physiological vasodilator of the coronary system (Gerlach et al., 1963).

Several lines of evidence suggest that this cycle does not operate in perfused rat heart at a rate sufficient to account for the observed

ammonia production. Although all the enzymes, when measured under saturating conditions (Maguire et al., 1972), are sufficiently active to account for the observed ammonia production, the intermediates adenosine, inosine, hypoxanthine and IMP cannot be found in rat heart tissue unless it is severely hypoxic (Gerlach et al., 1963) nor in rabbit heart unless it is hypoxic or uncoupled for 20 minutes or longer (Richman and Wyborny, 1964). There is evidence that adenosine given to perfused rabbit heart is converted to AMP (by adenosine kinase), rather than to inosine (Liu and Feinberg, 1971). Finally, the cycle implies turnover of the α-phosphate of AMP, but Rossi et al. (1972) found the rate of turnover to be only one twentieth of the rate of ammonia production as measured in this study.

5.2 Amino Acid Metabolism and Ammonia

Since adenine nucleotide breakdown is insufficient to account for the observed ammonia production in perfused rat heart, alternative precursors must be considered. Comparison of enzyme levels in heart and skeletal muscle suggests that deamination of glutamate is potentially more important than deamination of adenine compounds in heart, whereas the reverse is true in skeletal muscle (Table 5.2)

Direct deamination of glutamate by glutamate dehydrogenase provides a route for the deamination of amino acids linked to glutamate by transamination. Glutamine is another possible precursor of ammonia (Ottaway, 1969a,b), but is less important in the context of overall amino group metabolism since it may not be formed from other amino acids in heart (Iqbal and Ottaway, 1970).

In the present section, experimental results are presented which attempt to relate ammonia production or utilization to myocardial amino acid metabolism. The implication of the results, and problems arising from them, will be discussed in the final section of this chapter (Section 5.3).

5.2.1 Total Ninhydrin-Reactive Material

Definition "Total ninhydrin-reactive material" is an unambiguous, if cumbersome, phrase to indicate material which reacts with ninhydrin under the conditions described in Chapter 2 (Section 2.2.4) to give a colour absorbing light at 570 nm. It includes free amino acids, compounds such as taurine, ammonia and small soluble peptides such as glutathione. In this and subsequent chapters it will be abbreviated to NRM. Within this category it is possible to define amino nitrogen as

	Rat leg muscle	Rat heart
Glutamate dehydrogenase	1.3	11
AMP deaminase	127	1.6 - 4.4

Table 5.2 Concentration of ammonia-producing enzymes in rat heart and skeletal muscle (data from Lowenstein, 1972).

Units are μmol substrate converted per g tissue per min at 37°C.

Treatment	Total NRM, μmol/g fresh weight (mean ± S.E.M.)	
Unperfused, tissue	34.8 ± 1.9	(7 hearts)
Perfused, no added N source:		
Tissue	24.8 ± 2.3	(9 hearts)
Perfusate	14.9 ± 1.5	(9 hearts)
Total	39.7 ± 3.1	(9 hearts)

Table 5.3 Total NRM in tissue and perfusate before and after perfusion.

Treatment	Total NRM, µmol/g, in tissue (mean ± S.E.M.)	Significance	
		A	B
Unperfused	34.8 ± 1.9 (7)	-	-
Perfused, no added N source	24.8 ± 2.3 (9)	$P < 0.01$	-
Perfused + 300 µmol/l NH_4^+	32.7 ± 1.9 (4)	$P > 0.4$	$0.05 < P < 0.1$
Perfused + 500 µmol/l glutamine	33.8 ± 1.1 (5)	$P > 0.6$	$P < 0.02$

Table 5.4 The effect on tissue NRM of perfusion with various media.

The number of hearts in which NRM was measured is shown in parenthesis. Significance A refers to the difference between perfused and unperfused tissue; significance B refers to the difference between tissue perfused without an added nitrogen source and that perfused with ammonia or glutamine. Differences were compared using Student's t-test.

nitrogen covalently bound to carbon in the form of an amino group ($-NH_2$). The phrase "total amino nitrogen" thus defined does not include ammonia.

The NRM content of rat heart, before and after perfusion, is shown in Table 5.3. In the absence of an added nitrogen source, the heart lost NRM into the perfusing fluid. There was, however, a net gain by the whole system of 4.9 μmoles per g. It was not possible to demonstrate statistical significance of this increase ($P > 0.2$), though there was probably a real increase due largely to protein degradation and reflecting the imbalance between protein breakdown and synthesis (see Section 5.2.3.1). The hydrolysis of glutamine to glutamate and ammonia would also result in an increase in NRM.

Loss of NRM from the perfused tissue was abolished if ammonia or glutamine were added to the perfusing medium (Table 5.4). It was not practicable to measure NRM in the perfusate under these conditions.

5.2.2. Myocardial Amino Acids

Table 5.3 shows that the unperfused rat heart contains 34.8 μmoles of ninhydrin-reactive material per g tissue. To determine the nature of this material, perchlorate extracts from two hearts were analyzed for amino acid composition (Moore and Stein, 1954a). The results are shown in Table 5.5. With the exceptions given below, the data are the means of values from the two hearts. Threonine, asparagine and glutamine elute as a single peak in the procedure used; serine in sufficient quantity appears as a shoulder on the side of this peak, but was not seen in these analyses. Glutamine was measured enzymically: the other three amino acids were not determined, but asparagine is present

Legend to Table 5.5

The table shows the content of free amino acids in rat heart as measured by the techniques described in the text. Figures in parenthesis are the number of hearts in which analyses were carried out (values are means ± S.E.M.). Other values are the means of two determinations by amino acid analyser. Literature values are either taken directly from the source cited or calculated from the data of the authors as µmol/g fresh weight. *-These authors quote ammonia concentrations in the heart of approximately 10 µmol/g. This almost certainly represents the large ammonia peak found in all automatic analyses, which arises from contamination of the reagents used.

(A) Herbert et al., 1966

(B) Kuttner and Lorincz, 1969

(C) Morgan et al., 1971

(D) Scharff and Wool, 1965a

(E) Manchester and Wool, 1963

Compound	This Author, µmol/g	Literature values, µmol/g				
		(A)	(B)	(C)	(D)	(E)
Aspartate	1.14 ± 0.10 (9)	0.56	1.47	0.55	3.02	2.68
Glutamine	5.49 ± 0.66 (9)	5.99	7.61	5.83	-	5.29
Glutamate	4.46 ± 0.24 (9)	4.45	7.76	4.63	4.24	4.18
Alanine	0.99 ± 0.12 (6)	1.92	1.15	0.62	2.32	3.61
Ammonia	0.43 ± 0.03 (6)	-	-	-	*	*
Glycine	0.82	0.76	0.47	0.35	0.68	1.53
Lysine	1.07	0.83	0.89	0.52	0.61	1.09
Arginine	0.33	0.13	0.29	0.13	0.27	1.19
Valine	0.050	0.15	0.12	0.08	0.11	0.77
Methionine	0.024	0.08	0.06	0.03	0.06	0.53
Isoleucine	0.034	0.08	0.08	0.04	0.06	0.70
Leucine	0.033	0.18	0.14	0.06	0.12	1.23
Tyrosine	0.018	0.09	0.06	0.04	0.08	0.39
Phenylalanine	0.028	0.07	0.05	0.03	0.05	0.50
Histidine	0.045	0.17	0.05	0.11	0.11	0.55
Proline	0.040	-	-	-	-	1.01
Tryptophan	0.006	-	-	-	0.02	-
Taurine	11.75	-	24.5	-	35.4	7.54
"Peak (1)"	4.52	-	-	-	-	-
Glutathione	2.27	-	-	-	4.11	-
Unidentified	0.27	-	-	-	-	-
Total (calculated)	33.8	-	-	-	-	-
Total NRM (measured)	34.8 ± 1.9 (7)	-	-	-	-	-
Nutritional status of rats	Fasted	Fasted	Fasted	Fasted	Fed	Fed

Table 5.5 The Free Amino Acid Content of Rat Heart, before Perfusion.

only in low concentration in heart (Ottaway, 1972). Data for aspartate, glutamine, glutamate, alanine and ammonia are the means (± standard error) of enzymic measurements. Obviously much more weight can be placed on these values than on the remainder. Examples of analyses taken from the literature are shown in comparison.

Nine amino acids (valine to tryptophan in the Table) constitute a very small proportion of the total (0.28 μmoles per g altogether, or 0.83% of the ninhydrin-reactive material). Any increase in their concentration during perfusion can be ascribed to protein degradation (Section 5.2.3.2), and they are unlikely to have any large-scale metabolic importance other than in protein metabolism. No transamination of the branched-chain amino acids could be demonstrated during perfusion (Section 5.2.3.2). In most future sections these nine amino acids will be dealt with as a group ("other amino acids"). Another group comprises glutamate, aspartate, alanine and glutamine. These constitute 36% of the total NRM, or 75% of the amino acids which occur in proteins (cf Gailis and Benmouyal, 1973), and their concentration in tissue exceeds that in plasma by a factor of ten or more (Scharff and Wool, 1965a). They are closely related to intermediates of energy metabolism. It is to this group that ammonia metabolism is most likely to be related, through the action of glutamate dehydrogenase or glutaminase. Relatively large amounts of taurine, glycine, lysine, arginine and glutathione (Wendell, 1970) are also present. "Peak (1)" is a large peak of unidentified material eluting between taurine and glutathione. It was not identified, although its chromatographic position shows it to be strongly acidic in nature, possibly a sulphonated compound connected with taurine metabolism. The high concentration of lysine, an essential amino acid,

is remarkable, but it probably derives from the breakdown of muscle proteins, in which it is a common residue (Kominz et al., 1954; Morgan et al., 1971; Elzinga and Collins, 1973). The absence of a lysine tranaminase (Felig and Wahren, 1971; Felig, 1973) means that it can only disappear from the tissue by diffusion out of the muscle cells.

The analyses of Table 5.5 show an interesting variation in aspartate concentration between fed and fasted rats. According to Bowman (1966), the aspartate content of rat heart falls on perfusion with acetate, β-hydroxybutyrate or octanoate. These substances increase the level of acetyl CoA and accelerate its conversion to citrate. Thus the requirement for oxaloacetate is increased and is satisfied by aspartate. Low aspartate concentrations in hearts from fasted rats may therefore indicate an increased dependence of these hearts on the metabolism of fatty acids and ketone bodies.

5.2.3 The Effect of Perfusion on Myocardial Amino Acids

Perfusion for 75 minutes without an exogenous nitrogen source led to an increase in ninhydrin-reactive material in the system (Section 5.2.1). The effect of perfusion on the free amino acid composition of rat heart is shown in Table 5.6. Values for glutamate, aspartate, alanine, glutamine and ammonia are the means (± standard error) of enzymic measurements. Values for other compounds are the means of total amino acid analysis of tissue and perfusate samples from two hearts. Since protein turnover acts as a source and sink of amino acids, an attempt will be made to estimate the extent of net protein degradation, prior to evaluation of changes in amino acid content during perfusion.

Compound	Concentration, µmol/g heart					
	Unperfused, Tissue (A)	Perfused, Tissue	Perfused, Perfusate	Perfused, Total (B)	(B-A)	Significance of (B-A)
Aspartate	1.14 ± 0.10	0.49 ± 0.13	0.33 ± 0.06	0.82 ± 0.13 (10)	-0.32	P = 0.07
Glutamine	5.49 ± 0.66	4.50 ± 0.40	2.33 ± 0.33	6.83 ± 0.66 (6)	+1.34	P = 0.2
Glutamate	4.46 ± 0.24	2.75 ± 0.28	0.30 ± 0.02	3.05 ± 0.28 (6)	-1.41	P < 0.01
Alanine	0.99 ± 0.12	0.79 ± 0.15	1.33 ± 0.12	2.12 ± 0.25 (9)	+1.13	P < 0.01
Ammonia	0.43 ± 0.03	0.32 ± 0.04	1.70 ± 0.21	2.02 ± 0.24 (7)	+1.60	P < 0.001
Glycine	0.82	0.34	0.47	0.81	-0.01	
Lysine	1.07	1.09	0.22	1.31	+0.24	
Arginine	0.33	0.39	0.08	0.47	+0.14	
Other amino acids	0.28	0.28	1.18	1.46	+1.18	
Taurine	11.75	10.89	0.94	11.83	+0.08	
Glutathione	2.27	1.26	0.44	1.70	-0.57	
"Peak (1)"	4.52	5.38	0.42	5.80	+1.28	
Unidentified	0.27	0.27	0.11	0.38	+0.11	
Total (calculated)	33.8	28.8	9.8	38.6	+4.8	
Total NRM (measured)	34.8 ± 1.9	24.3 ± 2.3	14.9 ± 1.5	39.7 ± 3.1 (9)	+4.9	P > 0.2

Table 5.6 Free amino acid content of rat heart, after perfusion for 75 minutes without an added nitrogen source.

Total NRM, and compounds measured by enzymic assays, are given as mean ± S.E.M., with the number of hearts in parenthesis. Other values are the means of two determinations by amino acid analyser. The term "other amino acids" embraces those from valine to tryptophan in table 5.5. The final column shows the significance of the change in the amino acid in the system, computed by the t-test. Values for unperfused heart are taken from table 5.5.

5.2.3.1 Lysine and Protein Turnover

Heart muscle protein is in a state of continuous breakdown and resynthesis (Manchester, 1970). During perfusion without an added nitrogen source, the tissue is in a state of negative nitrogen balance and breakdown is more rapid than synthesis. A part of the increase in free amino groups is due to this (Section 5.2.1). Lysine is an essential amino acid, a specific transaminase for which is absent in peripheral tissue, so that the increase in lysine can be used as an indication of net protein breakdown (Felig and Wahren, 1971; Felig, 1973; Marliss et al., 1971). On a molar basis the lysine content of muscle protein is approximately 8% (Black and Bolling, 1951, quote 8% in beef muscle; Morgan et al., 1971, 11.1% in rat heart myosin; Kominz et al., 1954; 6.0% and 10.1% in actin and myosin respectively). Perfused rat hearts produced on average* 0.21 μ moles of lysine per g fresh weight in 75 minutes. This is equivalent to an increase of 2.6 μ moles per g in total free amino acids due to protein breakdown.

Using this value, the contribution of protein breakdown to the increase in each amino acid on perfusion can be predicted and the observed increase can be compared with it (Table 5.7). In the Table, the approximate percentage composition of heart muscle protein is calculated from the data of Morgan et al. (1971) and Kominz et al. (1954), assuming equal amounts of actin and myosin. The complete amino acid sequence of actin from rabbit skeletal muscle has recently been published (Elzinga and Collins, 1973). The calculated composition agrees well with that given by Kominz et al. (1954).

* 2 tissue samples, mean increase 0.02 μ moles per g, and 5 perfusate samples, mean increase 0.19 μ moles per g, were used to calculate the average increase in lysine in the system.

Amino acid	Average % in heart muscle protein (residues per 100 residues)	Increase on perfusion (μmol/g)		
		Predicted	Observed	Corrected (observed-predicted)
spartate	6	0.16	-0.32	-0.48
lutamine	4	0.10	+1.34	+1.24
lutamate	11	0.29	-1.41	-1.70
lanine	9	0.23	+1.13	+0.90
lycine	6	0.16	-0.01	-0.17
ysine	8	0.21	+0.21	-
rginine	5	0.13	+0.14	+0.01
aline	5	0.13	+0.22	+0.09
ethionine	3	0.08	+0.09	+0.01
soleucine	6	0.16	+0.14	-0.02
eucine	9	0.23	+0.25	+0.02
yrosine	3	0.08	+0.16	+0.08
henylalanine	3	0.08	+0.25	+0.17
istidine	2	0.05	+0.08	+0.03

Table 5.7 Protein breakdown and amino acid changes on perfusion without an added nitrogen source.

The increase in lysine is taken as an index of protein breakdown. Using the composition of muscle proteins given by Morgan et al. (1971) and Kominz et al. (1954), the predicted increase in each amino acid on perfusion, due only to protein breakdown, is calculated. This value is used to correct the observed amino acid changes for the effects of protein turnover.

The new data allow discrimation, hitherto impossible, between glutamate, aspartate and their amides. Actin glutamate is 28% amidated, aspartate 35%. At the present time only fragments of the myosin sequence are known (Hodges and Smillie, 1972a,b; Huszar and Elzinga, 1971; Huszar, 1972), but these give no reason to doubt that the figures for amides in myosin are similar to those for actin. The contents of aspartate, glutamate and glutamine given in Table 5.7 are calculated on this basis.

5.2.3.2 Metabolically Inert Amino Acids

Methionine, isoleucine, leucine and arginine showed increases on perfusion which are identical, within the limits of experimental error, to these predicted in Table 7. The increases can thus be attributed to protein breakdown and strengthen confidence in the use of lysine in the calculation of protein degradation. The increases in tyrosine, phenylalanine, histidine and valine are greater than predicted, although they are essential amino acids and synthesis of their carbon skeletons cannot occur. The increases are, however, quantitatively small, and probably do not differ significantly from the predicted values. Proline and tryptophan were not measured in the perfused heart. None of these amino acids is concentrated more than fivefold from the plasma by the tissue (Scharff and Wool, 1965a), and they are unlikely to have much metabolic importance other than in the context of protein synthesis. It is interesting that no disappearance of leucine, isoleucine or valine could be shown, despite reports that heart contains an active branched-chain amino acid transaminase (Cammarata and Cohen, 1950) and readily oxidizes

these amino acids if they are presented in the perfusing medium (Clarke, 1957; Buse et al., 1972). Glycine does not appear to be oxidized by heart; it is not metabolized to CO_2 (Lorber and Olsen, 1946), nor does it cause an increase in alanine production (Coulson and Hernandez, 1968). It is possible that the small decrease observed on perfusion represents some conversion to serine, which could not be detected.

5.2.3.3 Metabolically Active Amino Acids:

Glutamate, Aspartate, Alanine and Glutamine

These four amino acids are closely related to the pathways of energy metabolism, as the diagram shows (Fig 5.3). They showed changes on perfusion (table 5.7) which differed greatly from the predicted increases due to protein breakdown.

The decrease in glutamate is statistically significant ($P < 0.01$) and has been observed before (Hicks and Kerly, 1960; Gailis and Benmouyal, 1973). It could be a result of the recovery of the heart from temporary anoxia after the onset of perfusion, since the concentration of glutamate is reported to be high in anoxia (Williamson, 1966). Bowman (1966) reported a fall in glutamate followed by partial recovery during perfusion. The total asparatate concentration also decreased significantly ($P < 0.01$) if protein-derived aspartate is taken into account. This decrease was observed by Randle et al. (1970) during 6 minutes perfusion. It is at variance with the results of Bowman (1966) who found a marked rise in aspartate at the onset of perfusion, falling later to the in vivo level, and Gailis and Benmouyal (1973), who showed a rise in aspartate during perfusion of

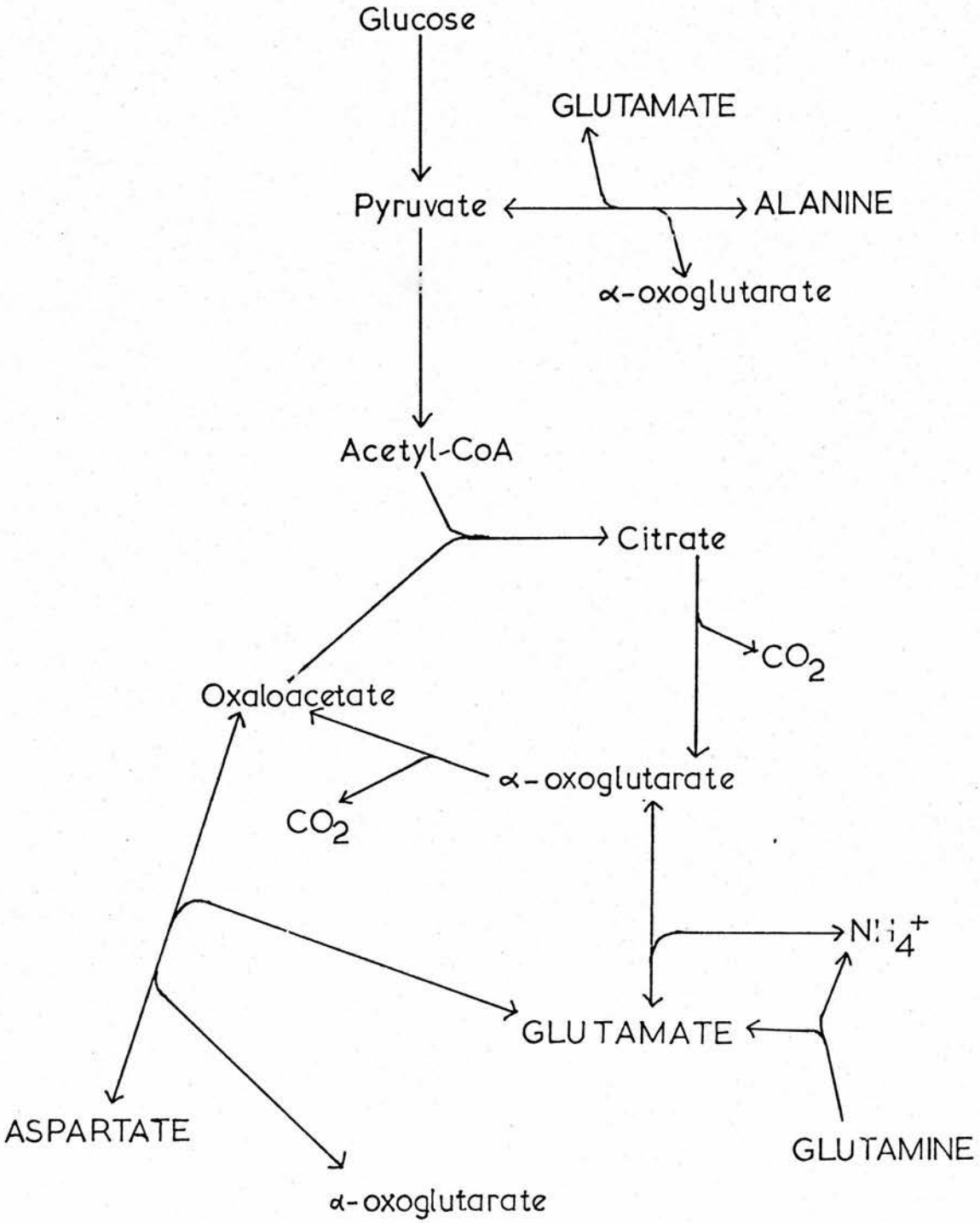


Figure 5.3. The relationship of glutamate, aspartate, glutamine, alanine and ammonia to energy metabolism.

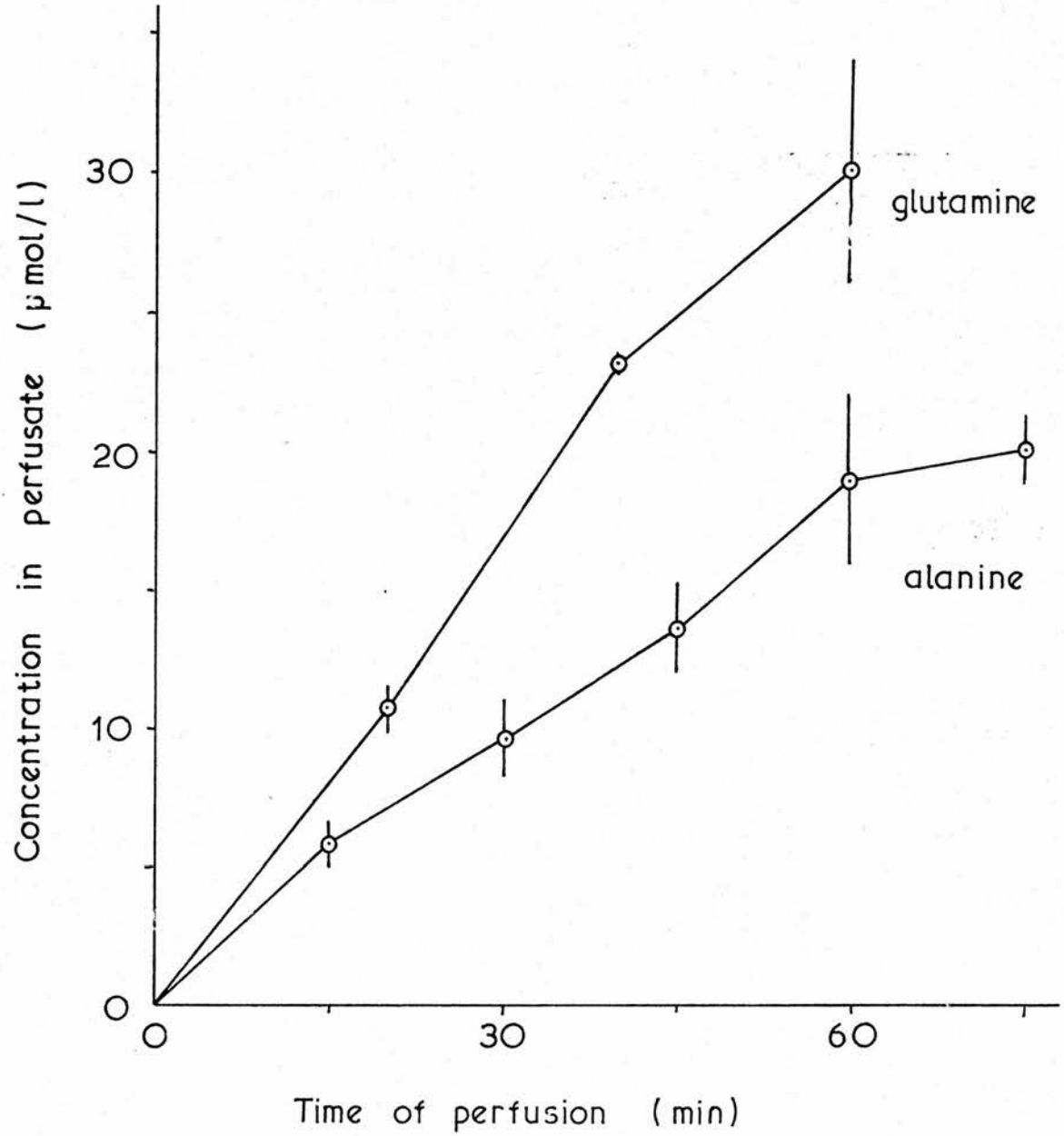


Fig. 5.4 The appearance of alanine and glutamine in the perfusate of hearts perfused without any additions.

Alanine and glutamine were measured in the perfusate of 4 hearts perfused without any added nitrogen source. Points are means, ± S.E.M.

guinea-pig heart. Hicks and Kerly (1960), on the other hand, found a complete disappearance of aspartate measurable chromatographically in two hours perfusion of rat heart.

In contrast to glutamate and aspartate, the total alanine concentration increased significantly ($P < 0.01$). An output of alanine is characteristic of peripheral tissues, particularly muscle (see Felig, 1973, for a review). The phenomenon is discussed in the context of heart amino-group metabolism in the final section of this chapter. The concentration of glutamine also increased on perfusion. Owing to the large experimental error involved in glutamine measurement, the increase was not statistically significant. It may not therefore be real, especially since glutamine synthetase has not been conclusively demonstrated in rat heart (see Chapter 1, Section 1.2). The fall in glutamine demonstrated by Ottaway (1969a) and Gailis and Benmouyal (1973) was not observed.

5.2.3.4 Perfusate Amino Acids

Analysis of the perfusate after 75 minutes (Table 5.6) showed that very little ninhydrin-reactive material diffused out of the heart other than ammonia, glutamine, alanine and taurine. Like ammonia (Chapter 3, Section 3.2.2), the rate of production of glutamine and alanine was linear, at least for the first 60 minutes of perfusion (Fig 5.4).

5.2.3.5 Nitrogen Balance

If protein degradation is taken into account, and the statistically insignificant increase in glutamine is ignored, the significant changes

are as follows:

Aspartate	- 0.48 µmol/g	Alanine	+ 0.90 µmol/g
Glutamate	- 1.70 µmol/g	Ammonia	+ 1.60 µmol/g
(Total)	- 2.18 µmol/g	(Total)	+ 2.50 µmol/g

The appearance of alanine and ammonia is thus almost accounted for by the disappearance of aspartate and glutamate. Considering the large errors involved, the agreement is remarkably good.

(If the observed increase in glutamine is taken into account, the nitrogen balance becomes seriously inexact, as can be seen from the following calculation. The synthesis of 1.24 µ moles of glutamine from glutamate and ammonia means an increase in NH_2 groups of 2.48 µ moles. Changes in the important amino acids in this case are as follows:

Aspartate	- 0.48 µmol/g	Glutamine	+ 2.48 µmol/g
Glutamate	- 1.70 µmol/g	Alanine	+ 0.90 µmol/g
		Ammonia	+ 1.60 µmol/g
(Total)	- 2.18 µmol/g	(Total)	+ 4.98 µmol/g

This result lends support to the view that there is no synthesis of glutamine in rat heart during perfusion, apart from that derived from protein breakdown).

5.2.4 Perfusion with Ammonia

Hearts perfused with ammonia (300 µ mol/l) in this series of experiments took up 2.9 µ moles of ammonia per g fresh weight from the perfusing medium. Only 0.27 µ moles of this can be accounted for

as an increase in the tissue concentration. The free amino acid compositions of tissue and perfusate are shown in Table 5.8.

Total alanine in these hearts was rather higher than in hearts perfused without ammonia, but it was not possible to show statistical significance (P = 0.22). The elevated tissue lysine concentration was probably due to chance biological variation. It is unlikely that it indicates a disturbance of protein synthesis by ammonia, since production of other "metabolically inert amino acids" (Section 5.2.3.2) was unaltered by the presence of ammonia, and the lysine content of the perfusate was similar to that found in the absence of ammonia. The predicted values for amino acids derived from protein breakdown are therefore the same as those used in Section 5.3.3 (Table 5.7).

The comparison between the predicted values and the changes observed on perfusion with added ammonia is given in Table 5.9. Again the increases shown by the "metabolically inert" amino acids differ little from the predicted values, whereas those shown by glutamate, aspartate and alanine differ greatly from the latter. Concentrations of these three amino acids in the system were significantly different from those in the unperfused heart, but not significantly different from those in the heart perfused without an added nitrogen source. The increase in glutamine was not significant, and indeed was less than that measured in the absence of added ammonia.

Nitrogen Balance

If, as before (Section 5.2.3.5), protein degradation is taken into account, and the increase in glutamine is ignored, the changes in the concentrations of important compounds are as follows (p. 192):

Compound	Concentration, µmol/g fresh weight								
	Unperfused, tissue (A)	Perfused, no added N source, total (B)	Perfused with 300 umol/l ammonia, Tissue	Perfusate	Total (C)	(C-A)	Significance of (C-A)	(C-B)	Significance of (C-B)
Aspartate	1.14 ± 0.10	0.82 ± 0.13	0.47 ± 0.11	0.32 ± 0.08	0.79 ± 0.10 (4)	-0.35	P = 0.06	-0.03	P > 0.9
Glutamine	5.49 ± 0.66	6.83 ± 0.66	4.69 ± 0.82	1.36 ± 0.08	6.05 ± 0.81 (8)	+0.56	P = 0.6	-0.78	P = 0.5
Glutamate	4.46 ± 0.24	3.05 ± 0.28	2.98 ± 0.22	0.23 ± 0.01	3.21 ± 0.22 (8)	-1.25	P < 0.01	+0.16	P > 0.6
Alanine	0.99 ± 0.12	2.12 ± 0.25	1.04 ± 0.25	1.63 ± 0.17	2.67 ± 0.38 (4)	+1.68	P < 0.001	+0.55	P = 0.2
Ammonia	0.43 ± 0.03	2.02 ± 0.24	0.70 ± 0.08	-2.88 *	-2.61 * (8)	-	-	-	-
Glycine	0.82	0.81	0.33	0.41	0.74	-0.08		-0.07	
Lysine	1.07	1.31	1.69	0.27	1.96	+0.89		+0.65	
Arginine	0.33	0.47	0.57	0.06	0.63	+0.30		+0.16	
Other amino acids	0.28	1.46	0.28	1.01	1.29	+1.01		-0.17	
Taurine	11.75	11.83	12.78	0.81	13.59	+1.84		+1.76	
Glutathione	2.27	1.70	1.92	0.45	2.37	+0.10		+0.67	
"Peak (1)"	4.52	5.80	4.84	0.43	5.27	+0.75		-0.53	
Unidentified	0.27	0.38	0.27	0.06	0.33	+0.06		-0.05	
Total (calculated)	33.8	38.6	32.6	7.04	39.6	+5.8		+1.0	
Total NRM (measured)	34.8 ± 1.9	39.7 ± 3.1	32.7 ± 1.9 (4)	-	-	-		-	

Table 5.8 Free amino acid content of rat heart after perfusion with 300 µmol/l ammonia.

Total NRM, and compounds measured by enzymic assays, are given as mean ± S.E.M., with the number of hearts in parenthesis. Other values are the means of two determinations by amino acid analyser. *-These figures are changes in concentration, not absolute values. Values for unperfused heart and heart perfused without added nitrogen are taken from Tables 5.5 and 5.6, respectively.

Differences were compared using Student's t-test.

Amino acid	Increase on perfusion (μmol/g)		
	Predicted	Observed	Corrected (observed-predicted)
Aspartate	0.16	-0.35	-0.51
Glutamine	0.10	+0.56	+0.46
Glutamate	0.29	-1.25	-1.54
Alanine	0.23	+1.68	+1.45
Glycine	0.16	-0.08	-0.24
Lysine	0.21	+0.89	+0.68
Arginine	0.13	+0.30	+0.17
Valine	0.13	+0.17	+0.04
Methionine	0.08	+0.08	0.00
Isoleucine	0.16	+0.11	-0.05
Leucine	0.23	+0.19	-0.04
Tyrosine	0.08	+0.10	+0.02
Phenylalanine	0.08	+0.23	+0.15
Histidine	0.05	+0.13	+0.08

Table 5.9 Protein breakdown and amino acid changes on perfusion with added ammonia.

The correction of observed amino acid changes is carried out as described in the legend to table 5.7. The apparent increase in lysine is due to high concentrations of lysine in the tissue of the two hearts analysed, probably reflecting biological variation.

Aspartate	- 0.51 µmol/g	Alanine	+ 1.45 µmol/g
Glutamate	- 1.54 µmol/g		
Ammonia	- 2.61 µmol/g		
(Total)	- 4.66 µmol/g	(Total)	+ 1.45 µmol/g

If the increase in glutamine is included in the calculation, the balance is:

Aspartate	- 0.51 µmol/g	Alanine	+ 1.45 µmol/g
Glutamate	- 1.54 µmol/g	Glutamine (as amino groups)	+ 0.92 µmol/g
Ammonia	- 2.61 µmol/g		
(Total)	- 4.66 µmol/g	(Total)	+ 2.37 µmol/g

Thus in the presence of ammonia, nitrogen balance is inexact whether glutamine is considered to increase or not. The net disappearance of nitrogen represented by the discrepancy in the first calculation is 3.21 µ moles per g tissue. This problem is discussed below (Section 5.3.2.3). The increases in lysine, taurine and glutathione shown in Table 5.8 cannot be ascribed to any process involving fixation of NH_3.

5.2.5 Perfusion with Added Amino Acids

5.2.5.1 Perfusion with Glutamine

Fig 5.5 shows the uptake of glutamine by perfused rat heart from a medium in which its concentration is 500 µ moles per litre. The net uptake (1.5 $\pm$ 2.1 µ moles per g tissue) was small and seemed to occur within the first 15 minutes of perfusion. Owing to the wide biological variation between the behaviour of different hearts

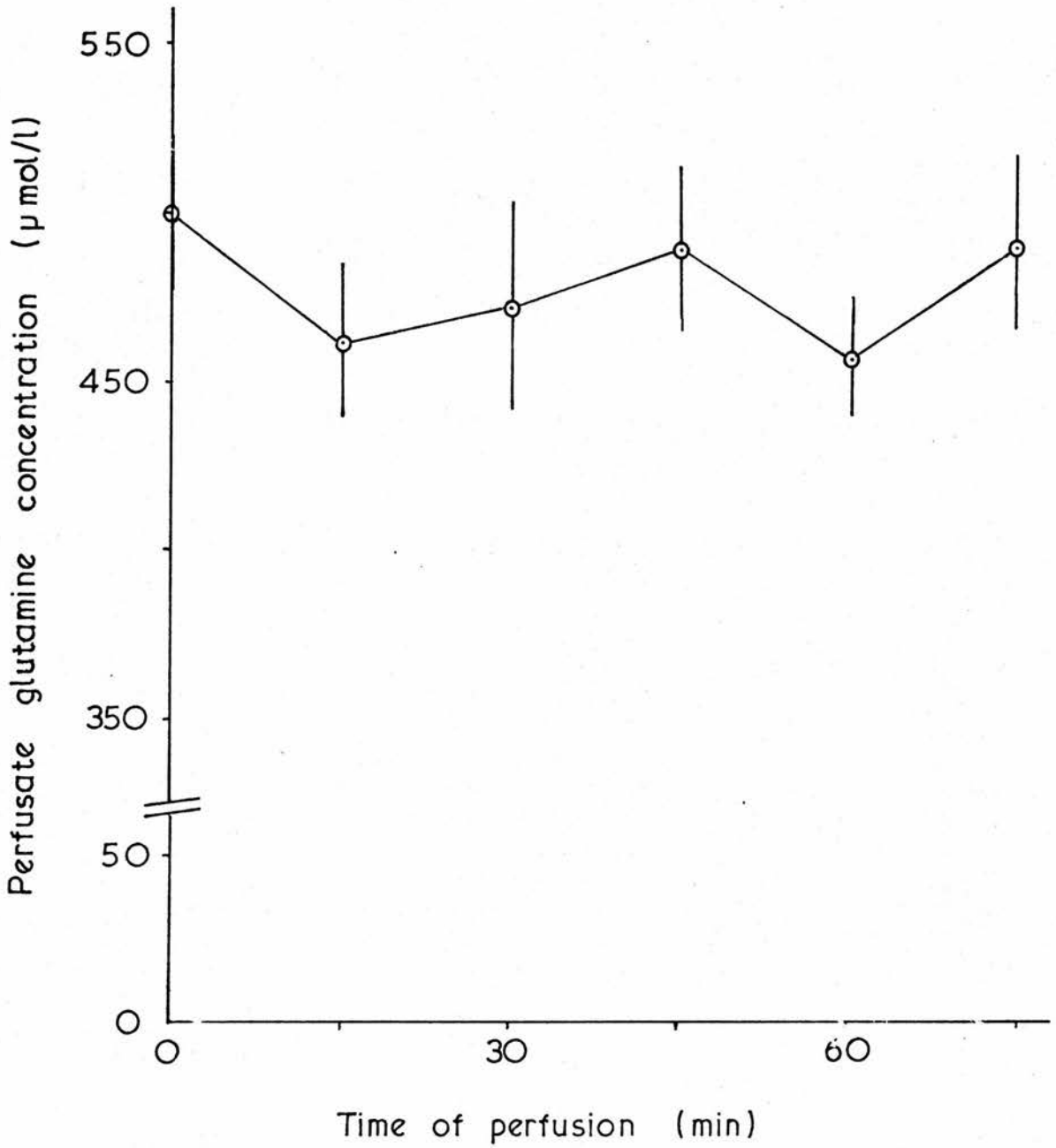


Fig. 5.5 The uptake of glutamine by perfused rat heart.

Points are means ± S.E.M. of serial glutamine measurements in the perfusate of 8 hearts.

Amino acid	Concentration, µmol/g						(D-A)	Significance of (D-A)	(D-B)	Significance of (D-B)
	Unperfused, tissue (A)	Perfused, no added N source, total (B)	Perfused with 500 µmol/l glutamine, Tissue	Perfusate	Total (D)					
Aspartate	1.14 ± 0.10	0.82 ± 0.13	1.48 ± 0.32	0.30 ± 0.11	1.78 ± 0.28	(5)	+0.64	P < 0.05	+0.96	P < 0.01
Glutamine	5.49 ± 0.66	6.83 ± 0.66	6.43 ± 0.97	-1.50 *	-0.56 *	(7)	-	-	-	-
Glutamate	4.46 ± 0.24	3.05 ± 0.28	2.75 ± 0.17	0.63 ± 0.17	3.38 ± 0.20	(4)	-1.08	P < 0.02	+0.33	P > 0.4
Alanine	0.99 ± 0.12	2.12 ± 0.25	1.16 ± 0.09	2.34 ± 0.40	3.50 ± 0.53	(5)	+2.51	P < 0.001	+1.38	P < 0.02
Ammonia	0.43 ± 0.03	2.02 ± 0.24	0.96 ± 0.18	2.57 ± 0.32	3.53 ± 0.43	(8)	+3.10	P < 0.001	+1.50	P < 0.02

Table 5.10 Free amino acid content of rat heart after perfusion with 500 µmol/l glutamine.

Values are means ± S.E.M., with the number of hearts in parenthesis.
*-These figures are concentration changes, not absolute values.

Differences were compared using Student's t-test.

Amino acid	Increase on perfusion (μmol/g)		
	Predicted	Observed	Corrected (observed-predicted)
Aspartate	0.16	+0.64	+0.48
Glutamine	0.10	-0.56	-0.66
Glutamate	0.29	-1.08	-1.37
Alanine	0.23	+2.51	+2.28

Table 5.11 Protein breakdown and amino acid changes on perfusion with added glutamine.

See legend to table 5.7.

it was not possible to demonstrate that this value was significantly different from zero. However, hearts perfused with glutamine produced significantly more alanine, ammonia and aspartate than those perfused without an added nitrogen source (Table 5.10), and these changes confirm that glutamine was taken up by the heart. The small change in glutamate was not significant ($P > 0.4$). Perfusion with glutamine was the only treatment which significantly increased the synthesis of ammonia by the heart ($P < 0.02$). It might be argued that ammonia arose from the spontaneous conversion of glutamine to ammonium pyrrolidonecarboxylate in the perfusate during the course of the experiment (Hamilton, 1945). However, the tissue ammonia concentration in hearts perfused with glutamine was three times that measured in hearts perfused without any added nitrogen source (Table 5.6; significance of increase in tissue ammonia, $P < 0.01$), and was indeed higher than that in hearts perfused with 300 μmol/l ammonia. These observations clearly show that the increased ammonia is produced in the tissue in response to the uptake of glutamine.

Using the values for the contribution of protein turnover to amino acid metabolism derived in Table 5.7, the corrected amino acid changes on perfusion can be calculated (Table 5.11) and the nitrogen balance can be presented as follows:

Glutamate	- 1.37 μmol/g	Aspartate	+ 0.48 μmol/g
Glutamine (as amino groups)	- 1.32 μmol/g	Alanine	+ 2.28 μmol/g
		Ammonia	+ 3.10 μmol/g
(Total)	- 2.69 μmol/g	(Total)	+ 5.84 μmol/g

Figures are μmoles per g fresh weight.

These figures show a discrepancy of 3.15 μmoles per g between the synthesis of aspartate, alanine and ammonia and the disappearance of glutamate and glutamine. In view of the biological variation in glutamine uptake, and the analytical error involved in measuring the small changes in perfusate glutamine concentration, some discrepancy is perhaps to be expected.

5.2.5.2 Perfusion with Glutamate

In contrast to glutamine, the uptake of glutamate by perfused rat heart was unequivocal and continued throughout the first hour of perfusion (Fig 5.6), during which time approximately one quarter of the added glutamate disappeared from the perfusate. The uptake from the perfusate was 7.9 ± 1.8 μmoles per g fresh weight, and was significantly greater than zero ($P < 0.02$). Hence of the compounds tested the uptake of glutamate was quantitatively the most important, confirming the results of Keul and co-workers (1964, 1966) in human heart.

Hearts perfused with glutamate produced more glutamine and significantly more aspartate ($P < 0.02$) than those perfused without an added nitrogen source (Table 5.12). An increase in alanine production was also noted in the one heart and two perfusates in which it was measured. It was not possible to demonstrate a significant increase in ammonia production by the whole system, although the tissue ammonia concentration was significantly raised ($P < 0.01$) over that in hearts perfused without an added nitrogen source, as was the tissue glutamine ($P < 0.001$).

Using the values for the contribution of protein turnover to amino acid metabolism derived in Table 5.7, the corrected amino acid

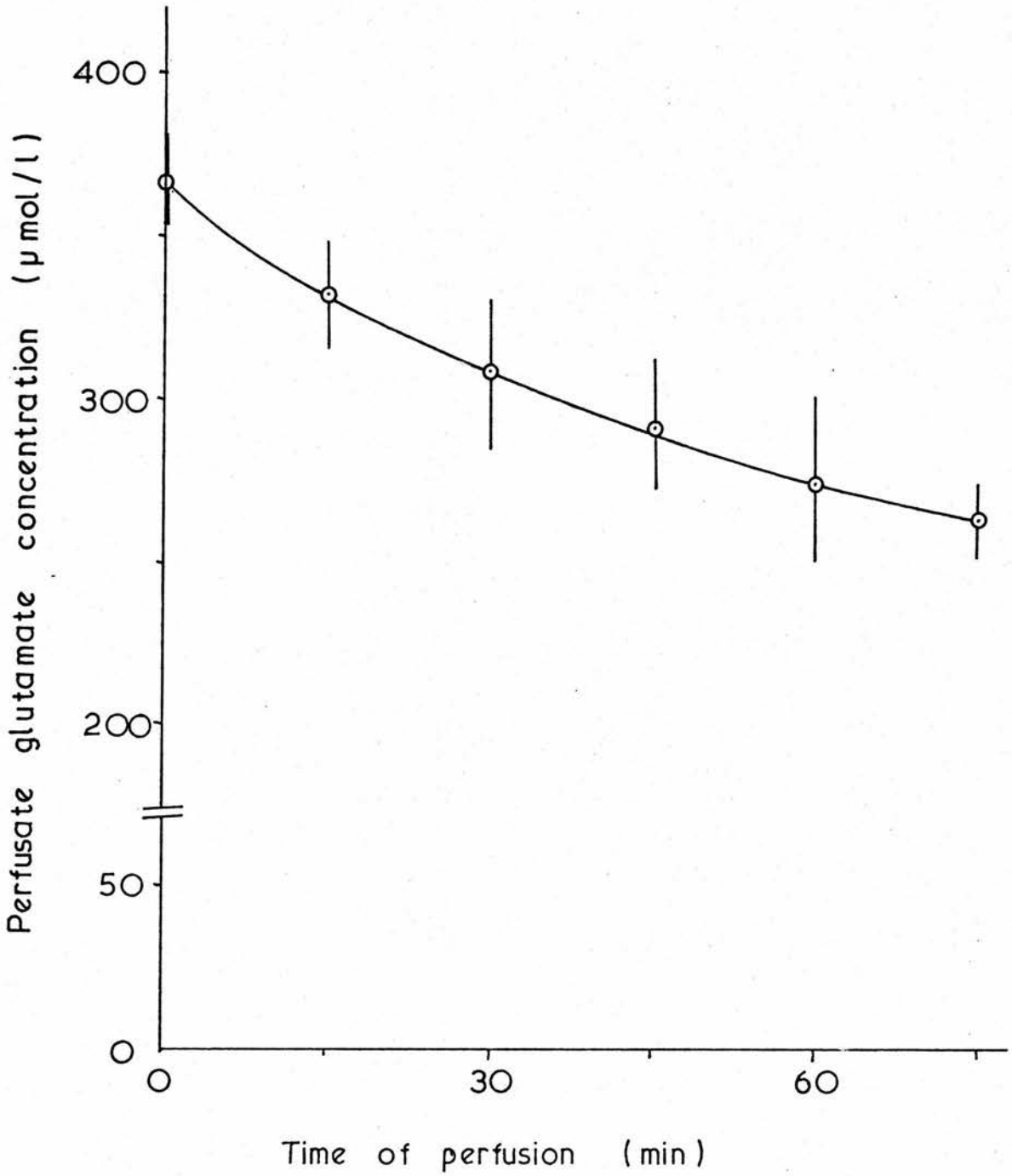


Fig. 5.6 The uptake of glutamate by perfused rat heart.

Points are means ± S.E.M. of serial glutamate measurements in the perfusate of 6 hearts.

Amino acid	Concentration, µmol/g heart					(E-A)	Significance of (E-A)	(E-B)	Significance of (E-B)
	Unperfused, tissue (A)	Perfused, no added N source, total (B)	Perfused with 370 µmol/l glutamate, Tissue	Perfusate	Total (E)				
Aspartate	1.14 ± 0.10	0.82 ± 0.13	0.77 ± 0.14	0.93 ± 0.17	1.70 ± 0.40 (4)	0.56	P = 0.07	0.88	P < 0.02
Glutamine	5.49 ± 0.66	6.83 ± 0.66	7.49 ± 0.29	1.63 ± 0.09	9.29 ± 0.51 (3)	3.80	P < 0.05	2.46	P = 0.09
Glutamate	4.46 ± 0.24	3.05 ± 0.28	2.69 ± 0.25	-7.90 *	-9.67 * (5)	-	-	-	-
Alanine	0.99 ± 0.12	2.12 ± 0.25	1.13 (1)	2.59 (2)	3.72 (1)	2.73	-	1.62	-
Ammonia	0.43 ± 0.03	2.02 ± 0.24	0.57 ± 0.06	1.51 ± 0.26	2.39 ± 0.37 (4)	1.96	P < 0.001	0.36	P > 0.4

Table 5.12 Free amino acid content of rat heart after perfusion with 370 µmol/l glutamate.

Values (with the exception of alanine) are means ± S.E.M., with the number of hearts in parenthesis. *-These figures are concentration changes, not absolute values.

Differences were compared using Student's t-test.

Amino acid	Increase on perfusion (μmol/g)		
	Predicted	Observed	Corrected (observed-predicted)
Aspartate	0.16	+0.56	+0.40
Glutamine	0.10	+3.80	+3.70
Glutamate	0.29	-9.67	-9.96
Alanine	0.23	+2.73	+2.50

Table 5.13 Protein breakdown and amino acid changes on perfusion with added glutamate.

See legend to table 5.7.

changes on perfusion were calculated (Table 5.13) and the nitrogen balance expressed as follows:

Glutamate	- 9.96 µmol/g	Aspartate	+ 0.40 µmol/g
		Glutamine	+ 7.40 µmol/g
		Alanine	+ 2.50 µmol/g
		Ammonia	+ 1.96 µmol/g
(Total)	- 9.96 µmol/g	(Total)	+ 12.26 µmol/g

Figures are µ moles per g fresh weight. They show a discrepancy of 2.30 µ moles per g between the nitrogen gained and the nitrogen lost.

5.2.5.3 Perfusion with Aspartate

The aspartate concentration in the perfusate of 3 hearts perfused with media containing aspartate (350 - 500 µ mol/l) remained constant for the first 60 minutes of perfusion, suggesting that aspartate is not taken up by the perfused heart during this period (Fig 5.7). The slight decrease in the perfusate aspartate concentration in two experiments from 60 to 75 minutes is unlikely to represent a sudden uptake of aspartate (see Section 5.3.5).

Some related metabolites were measured in these hearts (Table 5.14). Hearts perfused with aspartate liberated more ammonia into the perfusate than those perfused without any added nitrogen source, but the increase in the perfusate ammonia concentration was not significant ($P > 0.1$). There was no evidence of any increase in alanine production in the single heart in which this amino acid was measured. It seems unlikely therefore that aspartate was taken up by the perfused rat

heart, and extremely unlikely in the intact rat, where the plasma aspartate concentration is much lower (38 μ moles per litre as measured by Scharff and Wool (1965a) in fed rats) than that used in the experiments described in this section.

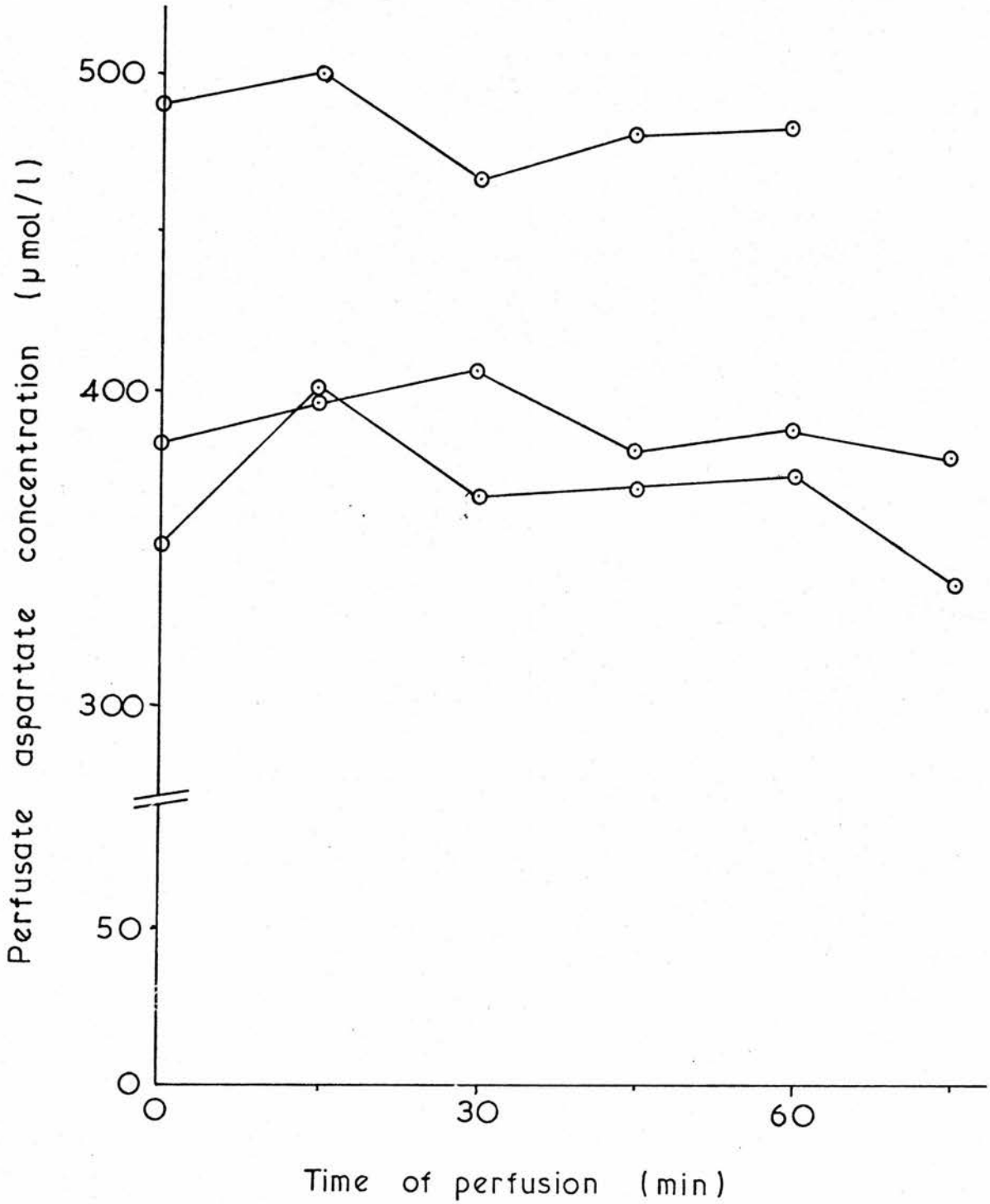


Fig. 5.7 The concentration of aspartate added to the perfusate of rat heart.

Points represent single determinations of aspartate in serial perfusate samples from 3 hearts.

Compound measured	Treatment of heart	Concentration, µmol/g heart		
		Tissue	Perfusate	Total
Ammonia	Unperfused	0.43 ± 0.03 (6)	-	-
	Perfused, no N source	0.32 ± 0.04	1.70 ± 0.21	2.03 ± 0.24 (7)
	Perfused + aspartate	0.67 (1)	2.42 ± 0.40 (3)	3.09 (1)
Alanine	Unperfused	0.99 ± 0.12 (7)	-	-
	Perfused, no N source	0.79 ± 0.15	1.33 ± 0.12	2.12 ± 0.25 (9)
	Perfused + aspartate	1.21 (1)	1.48 (1)	2.69 (1)

Table 5.14 Ammonia and alanine changes on perfusion with aspartate.

A limited number of hearts was perfused with aspartate. Numbers are shown in parenthesis.

5 3 Discussion

The results reported in the preceding section of this chapter describe an investigation into the changes in the amino acid content of rat heart during perfusion, and the relation of the amino acids with each other and with ammonia in heart metabolism. Many problems and points of interest raised by the investigation are the subject of discussion in the present section.

5.3.1 Amino Acid Metabolism in the Perfused Rat Heart

Total amino acid analysis has shown that the only amino acids whose concentrations change significantly on perfusion are glutamate, glutamine, aspartate and alanine (Section 5.2.3.3). Other amino acids show smaller concentration changes which can be related to protein breakdown in the tissue. The concentration of taurine, as measured in the present study and by other authors (Table 5.5), exceeds that of every other amino compound measured in rat heart. Its function in this tissue is as yet unknown, although a transmittory or excitatory role has been postulated for it (Huxtable and Bressler, 1972). It is unlikely to have any metabolic relationship with any of the four amino acids named above, or to ammonia. Glutathione was also present in relatively high concentration, but its concentration could not be measured accurately since its peak was overlapped on the chromatogram by those of taurine, which preceded it, and "peak 1", which followed it. Changes in its concentration reported in Tables 5.6 and 5.8 are not mirrored by changes in the glycine concentration, which suggests that the glutathione concentration in fact remains

Fig. 5.8 Concentrations of amino acids and ammonia in perfused rat heart.

The histogram shows the concentration of each compound as μmol/g fresh weight in tissue before perfusion and in tissue and perfusate after perfusion for 75 min. The stippled portion of each bar represents the tissue concentration, the blank portion the perfusate concentration. Thus the complete bar represents the total concentration in the system. Short vertical lines represent S.E.M.'s both of tissue concentration and of total concentration.

Treatment of the hearts was as follows:

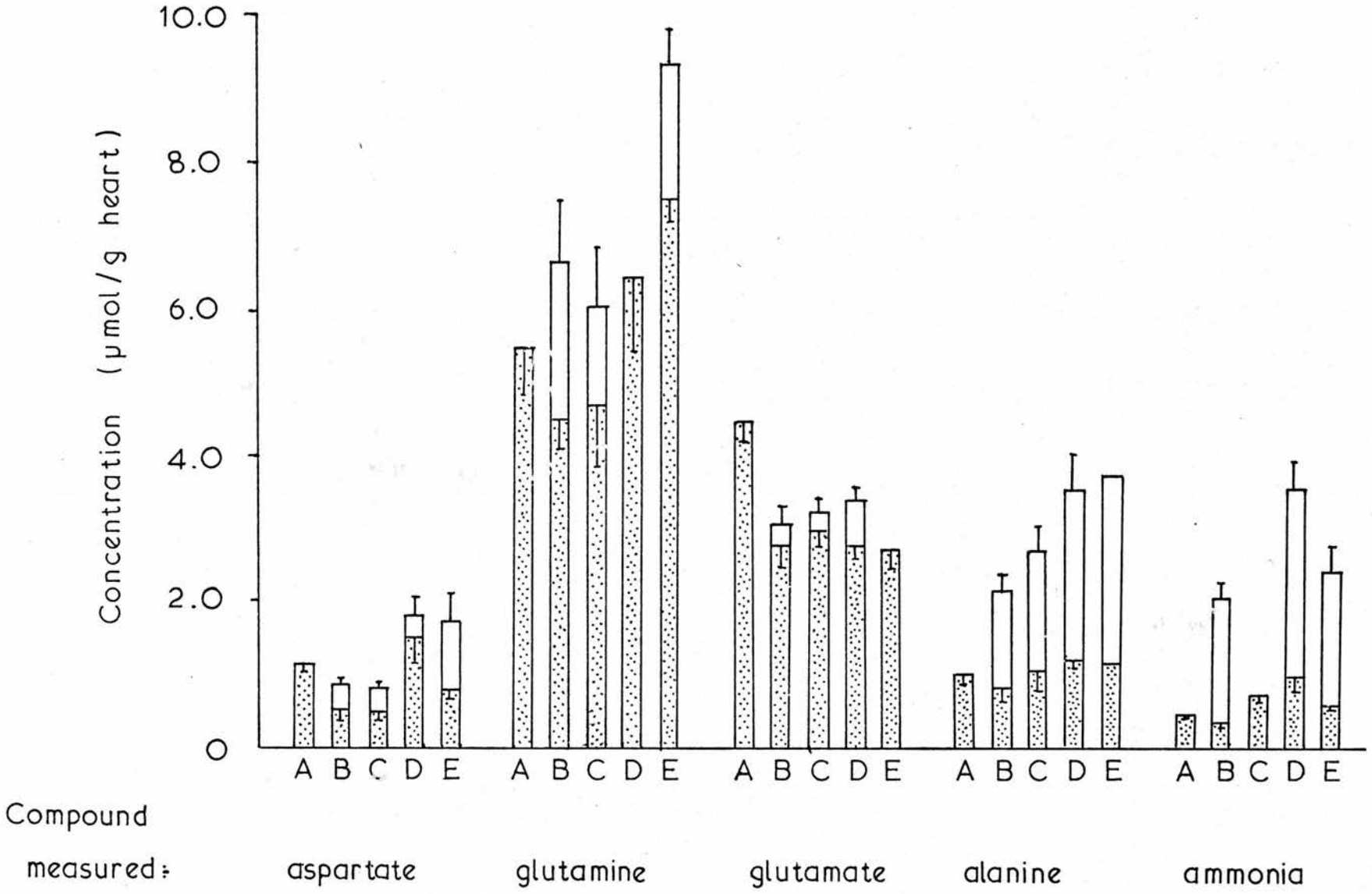
A Unperfused

B Perfused with no added nitrogen source

C Perfused with 300 μmol/l ammonium chloride

D Perfused with 500 μmol/l glutamine

E Perfused with 370 μmol/l glutamate.


10.0
8.0
6.0
4.0
2.0
0
Concentration (μmol/g heart)
A B C D E
A B C D E
A B C D E
A B C D E
A B C D E
Compound measured:
aspartate
glutamine
glutamate
alanine
ammonia

constant during perfusion. This discussion will therefore be confined to glutamate, glutamine, aspartate, alanine and ammonia.

Figure 5.8 shows the concentration of these five compounds as μ mol/g fresh weight in the tissue of unperfused hearts, and in the tissue and perfusate of hearts after perfusion with the media described in the foregoing section.

5.3.1.1 The Metabolism of Endogenous Amino Acids

Perfusion without an added nitrogen source resulted in some disappearance of glutamate and aspartate, with production of alanine, ammmonia and possibly glutamine. (The calculation of Section 5.2.3.5 shows that nitrogen balance is inexact, but the discrepancy can be reduced if one assumes that no glutamine is synthesized). A possible explanation is that the net conversion of aspartate to alanine by the coupling of GOT to GPT results also in a net conversion of pyruvate to oxaloacetate, and is a mechanism for replenishing the tricarboxylic acid cycle in a tissue which lacks pyruvate carboxylase (Safer and Williamson, 1973):

(1) Aspartate + α-oxoglutarate ⟶ oxaloacetate + glutamate

(2) Glutamate + pyruvate ⟶ α-oxoglutarate + alanine

Sum: Aspartate + pyruvate ⟶ oxaloacetate + alanine

In the perfused rat heart, however, this type of anaplerotic reaction would seem to be unnecessary. In contrast to the substrate-depleted hearts of Safer and Williamson (1973), hearts taken straight from rats without anoxia and perfused with media containing glucose have

their full complement of tricarboxylic acid cycle intermediates (Bowman, 1966). In fact, Bowman's results suggest that the utilization of fat-related substances, as would occur during fasting, tends to increase rather than deplete the concentrations of these intermediates. The heart of a fasted rat, therefore, ought to have an excess rather than a deficiency of tricarboxylic acid cycle intermediates.

Moreover, this conversion does not explain the net disappearance of glutamate and the production of ammonia. Safer and Williamson (1973) did not detect any ammonia production, since tissue ammonia does not increase on perfusion (Chapter 3), and they did not measure ammonia in the perfusate. An alternative explanation is that glutamate and aspartate are oxidized to provide energy, the amino groups leaving the heart as alanine and ammonia. The "alanine cycle" (Felig, 1973) is discussed below (Section 5.3.3).

The nitrogen balance calculation of Section 5.2.3.5 shows a disappearance of 1.70 μ moles of glutamate and 0.48 μ moles of aspartate, and a production of 0.90 μ moles of alanine and 1.60 μ moles of ammonia per g fresh weight in 75 minutes of perfusion. If these figures represent amino acids utilized as a source of energy, the amount is insignificant in comparison with the utilization of carbohydrate energy sources. The hearts used in this study took up on average 30.9 μ moles of glucose per g per hour, producing 11.8 μ moles of lactate and 0.8 μ moles of pyruvate (Chapter 2) and presumably oxidizing most of the remainder by the tricarboxylic acid cycle, since no significant increase in tissue glycogen is seen on perfusion of hearts from fasted rats (Mowbray, 1969). Oxygen consumption was 3.61 μ moles per minute, or 309 μ moles per g per hour in a heart weighing 0.7 g (Table 2.5). Thus endogenous amino acids are not an

important source of energy in perfused rat heart which is supplied with glucose, confirming the findings of Keul and co-workers (1964, 1966) in human heart.

5.3.1.2 The Metabolism of Amino Acids in the Perfusate

Hearts took up nitrogenous compounds selectively from the perfusing fluid. No aspartate was taken up, at least in the first hour of perfusion. The uptake of ammonia was small, and caused an increase only in alanine production, but this increase was not statistically significant. Ammonia uptake from media of varying ammonia concentration has been fully documented in an earlier chapter (Chapter 3). The uptake of glutamine was smaller than that of ammonia, and was not easy to demonstrate in all hearts tested. It did, however, result in a significant increase in the synthesis of aspartate, alanine and ammonia compared with hearts perfused without any added nitrogen source. The extra alanine and ammonia formed were released by the heart into the perfusate. The extra aspartate, however, did not leave the heart, but accumulated in the tissue: the quantity of aspartate appearing in the perfusate was no different from that in hearts perfused without any added nitrogen source.

Glutamate was the only compound tested which was metabolized by the heart to an appreciable extent. Perfusion with glutamate caused an increased production of aspartate and alanine. The synthesis of glutamine by these hearts was significant, which suggests that rat heart may not, after all, lack glutamine synthetase (Iqbal and Ottaway, 1970). Ammonia production was not significantly increased by perfusion with glutamate; this treatment did, however,

cause a significant increase ($P < 0.01$) in the intracellular ammonia concentration. It is probable therefore that the ammonia formed from glutamate was used in glutamine synthesis and was not released into the perfusate. Although the increase in aspartate synthesis was almost identical to that caused by perfusion with glutamine, during perfusion with glutamate the greater part of the aspartate was released into the perfusate. Hence the output of aspartate into the perfusate was significantly raised ($P < 0.01$) by perfusion with glutamate, but was unaffected by any other treatment described, which suggests that aspartate was leaving the heart in exchange for some of the glutamate taken up. Glutamate-aspartate exchange has been observed in mitochondria (Chappell, 1968).

Work on human subjects shows that glutamate is taken up by the heart from the circulation, but that it does not contribute significantly to the heart's energy supply (Keul et al., 1964). The present study has shown that rat heart takes up glutamate from the perfusing medium, in which its initial concentration is 370 µ mol/l. Although the concentration of glutamate in the plasma of the fed rat is somewhat lower (113 µ mol/l, Scharff and Wool, 1965a), it is likely that uptake also occurs in the live animal. Hearts perfused with glutamate metabolized 9.96 µ moles of the amino acid per g tissue; although this figure is nearly six times greater than the metabolism of endogenous glutamate (1.70 µ mol/g), it still represents an insignificant source of energy when compared with the oxygen consumption of the heart (309 µ mol/g per hour). Peripheral tissues in general consume glutamate and release alanine; functions of this cyclic process will be discussed below (Section 5.3.3).

5.3.2 Amino Acid Metabolism and Ammonia

The production of ammonia from adenine nucleotides in rat heart was discounted in section 5.1 of this chapter. In section 5.2, an attempt was made to relate the synthesis or disappearance of ammonia in perfused rat heart to changes in the concentration of glutamate, glutamine, aspartate and alanine.

5.3.2.1 Ammonia Production

It was shown in section 5.2.5 that glutamine alone of the amino acids tested caused a significant increase in the production of ammonia. This does not necessarily mean that the ammonia produced in perfusion without an added nitrogen source comes exclusively from glutamine. It merely demonstrates the removal of an administered glutamine load, as expected, by the action of glutaminase (Ottaway, 1969a,b).

Hearts perfused with glutamate did not produce any more ammonia than those perfused with the control medium (containing no nitrogen source). However, the tissue ammonia concentration was significantly increased, and there was a significant synthesis of glutamine. This implies that glutamate did cause an increase in ammonia synthesis, but that the extra ammonia was used in the production of glutamine, instead of appearing in the perfusate.

Hearts perfused with no added nitrogen source produced 1.6 μ mol of ammonia per g tissue, but its source is more difficult to determine, although the experiments described above showed that ammonia could arise either from glutamate or from glutamine if these amino acids were administered in excess. The decrease in glutamate on perfusion with control media was 1.7 μ mol/g, which was almost identical to the

amount of ammonia synthesized. However, the ammonia formed during perfusion with the control medium appeared in the perfusate without accumulating in the tissue, whereas perfusion with glutamate caused no increase in the quantity of ammonia liberated into the perfusate. Endogenous glutamate is therefore a possible source of ammonia only if it is metabolized in a compartment different from that in which exogenous glutamate is metabolized. Glutamine has been proposed as a source of ammonia in rat heart, since this tissue contains glutaminase (Ottaway, 1969a,b) and the kinetic constants of this enzyme can be used to predict, with some success, the behaviour of isotopically labelled ammonia during perfusion (Chapter 4). This could not be substantiated by the amino acid measurements described here, since no decrease in glutamine was seen on perfusion with control media.

5.3.2.2 Ammonia Uptake

In the "nitrogen balance" calculation of section 5.2.4 it was not possible to account for the uptake of ammonia by hearts perfused with 300 μmol/l ammonia as an increase in bound amino groups, nor was it possible to demonstrate significant increases in any amino acid measured (although analysis of a greater number of hearts might conceivably have shown a significant increase in alanine). The problem of the fate of the ammonia nitrogen remains thus unsolved. The end product is unlikely to be glutamate, since no increase in the glutamate concentration of tissue or perfusate was caused by perfusion with ammonia, and perfusion with ^{15}N-labelled ammonia did not result in glutamate becoming significantly labelled (Chapter 4,

Section 4.3). Synthesis of adenine compounds is ruled out since there is insufficient inosine and IMP in heart to react with all the ammonia taken up (Gerlach et al., 1963). No significance can be attached to the apparent increase in taurine (Section 5.2.4) in ammonia-perfused hearts, since only two hearts were analyzed in each case, and it is difficult to see what connection taurine might have with ammonia metabolism. Metabolic uptake of ammonia by the heart has been attributed to the synthesis of glutamine (Watanabe, 1968), despite the failure of many authors to measure glutamine synthetase in rat heart (Iqbal and Ottaway, 1970; see also Chapter 1). In the present study, perfusion with ammonia did not increase glutamine formation in the heart (Section 5.2.4); nevertheless, perfusion with glutamate did lead to a significant production of glutamine which implies that glutamine must indeed be formed from ammonia and glutamate in rat heart.

5.3.2.3 Ammonia and Nitrogen Balance

In section 5.2 of this chapter, nitrogen balance calculations were presented for hearts perfused with the control medium and with media containing ammonia, glutamine or glutamate. Only those amino acids which were considered metabolically important by virtue of their high concentration in the tissue, significant concentration change on perfusion, and direct relationship with energy metabolism, were included in the calculations. All the calculations except that relating to the control medium showed discrepancies. The control calculation could be made approximately correct by assuming that no glutamine synthesis took place, but the observation that glutamine

Medium	Change in concentration, system, µmol/g					Total	Total (excluding ammonia)
	Ammonia	Aspartate	Glutamate	Alanine	Glutamine X2		
Control (no N source)	+1.60	-0.48	-1.70	+0.90	+2.48	+2.80	+1.20
Ammonia, 300 µmol/l	-2.61	-0.51	-1.54	+1.45	+0.92	-2.29	+0.32
Glutamine, 500 "	+3.10	+0.48	-1.37	+2.28	-1.32	+3.15	+0.05
Glutamate, 370 "	+1.96	+0.40	-9.96	+2.50	+7.40	+2.30	+0.34

Table 5.15 Nitrogen balance on perfusion with various media.

Figures show the gain or loss of metabolite from the system (tissue + perfusate) and are taken from the nitrogen balance calculations given in earlier sections of this chapter.

synthesis occurred in hearts perfused with glutamate suggests that this assumption is invalid. On perfusion with added ammonia the discrepancy involved a loss of nitrogen; perfusion with other media resulted in a gain of nitrogen.

Table 5.15 shows that all the calculations can be made more exact by omitting ammonia from the balance. This suggests that the ultimate source or fate of ammonia may not lie in the four amino acids measured, although it does not exclude any of these four amino acids from being either the direct precursor of ammonia, or the product formed directly from ammonia taken up by the heart.

5.3.3 Amino Acid Cycles in the Plasma of the Rat

Peripheral tissues, particularly skeletal muscle, release a large number of amino acids into the plasma (Pozefsky et al., 1969; Felig and Wahren, 1971; Cahill et al., 1972), amongst which alanine (Coulson and Hernandez, 1968; Pozefsky et al., 1968; Felig et al., 1970; Felig and Wahren, 1971) and glutamine (Marliss et al., 1971; Ruderman and Lund, 1972) are released in the largest quantity. Heart muscle likewise releases amino acids into the plasma (Keul et al., 1964); again alanine (Carlsten et al., 1961; Ottaway, 1969a) and glutamine (Keul et al., 1964) are the most important. Glutamate, in contrast, is taken up by forearm muscle (Marliss et al., 1971) and heart (Keul et al., 1964). The splanchnic circulation conversely removes alanine (Felig and Wahren, 1971), glutamine and other amino acids from the blood and produces glutamate (Marliss et al., 1971). Alanine (Mallette et al., 1969; Ishikawa et al., 1972) and glutamine (Ross et al., 1967) have been shown to act as substrates for

gluconeogenesis in the liver, and the availability of alanine seems to be rate-limiting for gluconeogenesis in this organ (Mallette et al., 1969). The alanine output of muscle is correlated with muscle activity and with the arterial pyruvate concentration (Felig and Wahren, 1971), and is increased by the administration of amino acids (Coulson and Hernandez, 1968). An "alanine cycle" has been proposed (Mallette et al., 1969; Felig et al., 1970; Felig and Wahren, 1971; Felig, 1973). One of its functions is the return of amino groups to the liver, following the oxidation by the muscle of amino acids, either derived endogenously from protein degradation or the ICF pool, or taken up from the plasma (as glutamate). In the liver, the carbon skeleton is converted via pyruvate to glucose which is returned to the plasma, the amino group being released as urea or glutamate (Fig 5.9). The cycle is completed by the return of glucose to the heart; however, the output of glutamate by the liver (Marliss et al., 1971) and its uptake by the heart (Keul et al., 1964) and by muscle (Marliss et al., 1971) provide an additional link in the cycle which is not discussed by the authors cited above. In addition to glutamate, leucine is reportedly taken up by muscle and metabolized, the nitrogen appearing as glutamine (Ruderman and Lund, 1972).

Glutamine has also been proposed as a nitrogen transporter (Braunstein, 1947; Ruderman and Lund, 1972), but its role is different from that of alanine, firstly since splanchnic uptake of glutamine is a function of the gut rather than the liver (Felig, 1973) and secondly since its output by peripheral tissues does not increase with activity (Felig, 1973) and is thus presumably unaffected by the rate of amino acid oxidation. It can only be of importance in tissues

in which significant glutamine synthesis can occur, for example in skeletal muscle and brain.

The metabolism of the perfused rat heart was in agreement with the above scheme. Alanine, glutamine and ammonia were released into the perfusate; other amino acids appeared in much smaller amounts (Section 5.2.3.4). The relative importance of alanine, glutamine and ammonia in disposing of amino groups added to the perfusing fluid is shown in Table 5.16. Values represent the output of each compound into the perfusate during perfusion, expressed as μmol/g tissue. Alanine synthesis seemed the most important mechanism for dealing with a nitrogen load, since the output of alanine was increased in response to the administration of amino compounds such as glutamate, glutamine or ammonia. The glutamine output showed no such increase however, and may represent merely a leakage of the amino acid from the large ICF glutamine pool. Results reported in section 5.2.5.2 did suggest that glutamine could be synthesized by the heart in response to the administration of glutamate, but there was no resultant increase in the output of glutamine into the perfusate under these conditions. Thus the physiological significance of glutamine output by the heart is uncertain. It does, however, constitute the major loss of amino acid nitrogen from the heart, as it carries two amino groups per molecule.

Ammonia output has never been considered as a significant method of amino group disposal, in view of its supposed toxicity (see Chapter 1, Section 1.3). Table 5.16 shows that it is quantitatively as important as alanine in the removal of nitrogen in control perfusions. However, like glutamine, it shows no increase in output in response to glutamate administration. The increase in output in response to glutamine is due to the action of glutaminase (Ottaway,

1969a,b). Aspartate is included in Table 5.16, although it does not represent a significant loss of amino groups in perfusion with ammonia, glutamine or the control medium. On perfusion with glutamate however, the quantity of aspartate released into the perfusate increased significantly ($P < 0.01$, compared to the aspartate output in control perfusions). The increase in the efflux of aspartate from the heart suggests that glutamate-aspartate exchange is involved to some extent in the uptake of glutamate.

The large uptake of glutamate relative to the amounts of the other compounds taken up (Section 5.2.5.2) is in agreement with the observations of Keul et al. (1964) in human subjects. Glutamate is a readily available and easily taken up source both of nitrogen and of carbon skeletons (Fig 5.9). It acts as a source of the amino groups of non-essential amino acids which are not themselves taken up from the plasma, for example aspartate. It acts as a source of tricarboxylic acid cycle intermediates in a tissue which lacks pyruvate carboxylase (Safer and Williamson, 1973) and so cannot derive these intermediates from pyruvate. It can be oxidized to provide energy, as long as the products, for example α-oxoglutarate or malate, can leave the heart at a rate sufficient to avoid accumulation. Such oxidation is accompanied by a stoicheiometric release of nitrogen in the form of alanine, aspartate and ammonia. It has been shown, however, (Section 5.3.1) that the energy provided by glutamate oxidation does not constitute a significant part of the heart's total energy requirement under normal circumstances, although it may become important when other sources of energy are in short supply, for example in starvation.

Added in perfusate	Uptake of compound added, µmol/g	Perfusate output (µmol/g) of:			
		Alanine	Ammonia	Glutamine	Aspartate
No addition	0.0	1.33	1.70	2.33	0.33
300 µmol/l NH_4 Cl	2.9	1.63	-	1.36	0.32
500 µmol/l glutamine	1.5	2.34	2.57	-	0.30
370 µmol/l glutamate	7.9	2.59	1.44	1.63	0.93

Table 5.16 The output of amino acids and ammonia in response to perfusion with various media.

The amount of each substance in the perfusate of rat heart after perfusion for 75 min is expressed as µmol per g fresh weight.

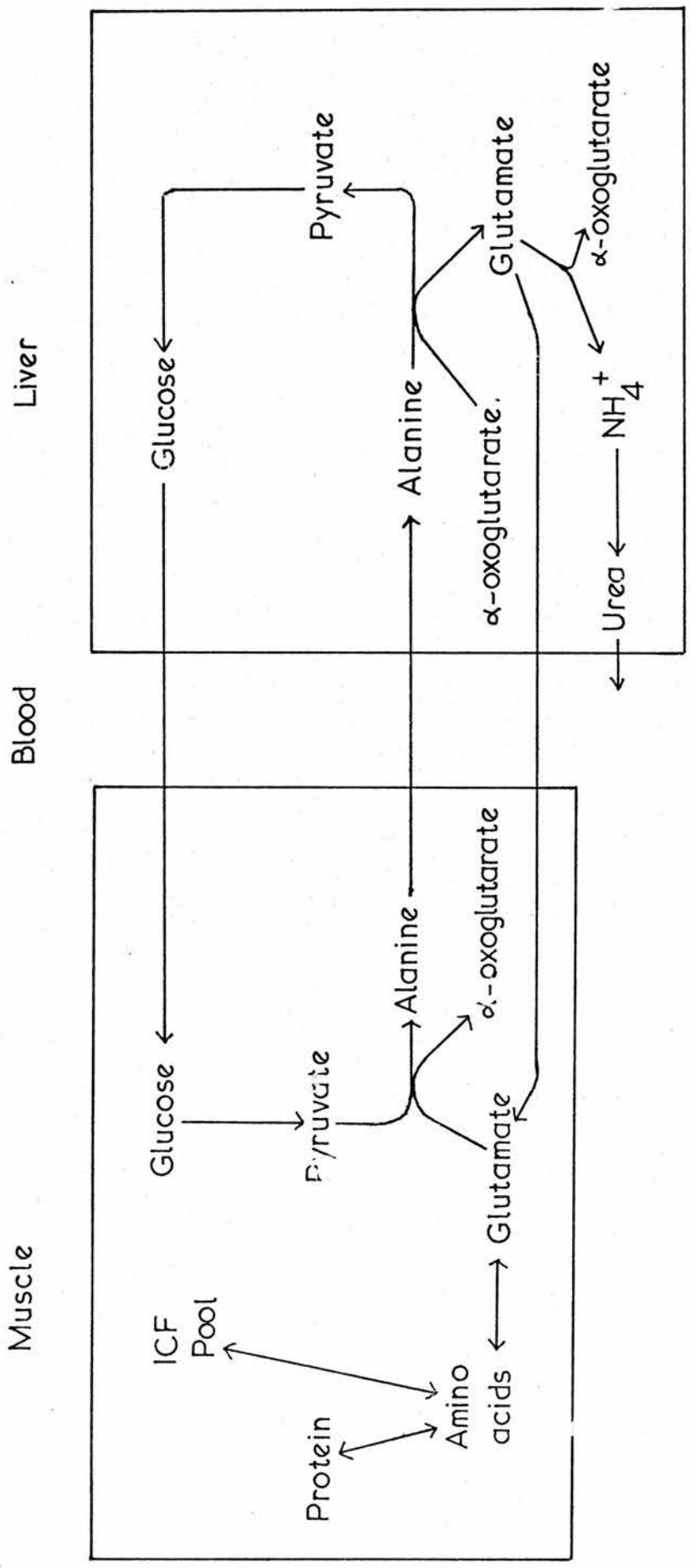


Figure 5.9. The glucose-alanine-glutamate cycle.

5.3.4 The Glutamate Concentration in Rat Heart

The glutamate concentration in the unperfused rat heart is remarkably constant at approximately 4.5 μmol/g fresh weight, as is shown by the agreement between the values given by various authors (Table 5.5, ignoring the value of Kuttner and Lorincz, 1969), despite variations in the concentrations of other amino acids such as alanine, glycine or aspartate. On perfusion with no added nitrogen source the tissue concentration fell to 2.75 μmol/g (Section 5.2.3.3). This value was not significantly affected by perfusion either with ammonia or with glutamine, or even with glutamate itself, despite the uptake of 7.9 μmol of glutamate per g of heart.

Thus there appears to be rigorous control of the glutamate concentration in rat heart, and the concentration at which it is controlled depends on the conditions: in these experiments there is a distinction between hearts of intact starved rats and hearts perfused with glucose medium. Williamson has observed that the steady-state concentration of glutamate depends on the status of the heart. The glutamate concentrations calculated from his data as μmol per g fresh weight were 4.5 in glucose perfusion and 1.4 in substrate-free perfusion (Safer and Williamson, 1973), and 6.3 in anoxia (Williamson, 1966). The value of 2.75 μmol per g reported here after glucose perfusion is rather lower than that of Safer and Williamson, who used fed rats and included insulin with glucose in the medium. His hearts were thus more dependent on glucose oxidation than were those used in the present investigation, and took up 72 μmol of glucose per hour per g fresh weight (Safer and Williamson, 1973) compared with the value of 30.9 reported in Chapter 2. It is

possible therefore that the glutamate content of the heart is affected in some way by the glycolytic flux. This does not, however, explain the high glutamate concentration in the unperfused heart, since one would expect the glycolytic flux to be relatively low in the starved animal.

It is more probable that the glutamate concentration in heart muscle reflects the mitochondrial NAD^+/NADH ratio, as it does in rat liver (Krebs, 1967; Williamson et al., 1967). Hence the high glutamate concentration in hearts perfused with fat-like substances such as acetate or octanoate (Bowman, 1966) or in anoxia (Williamson, 1966) may be due to a decrease in the NAD^+/NADH ratio. The decrease seen on perfusion could therefore represent recovery of the heart either from mild anoxia or from a fasting type of metabolism. However, the literature values of Table 5.5 are too uniform to suggest anoxia; and the values for hearts from fed rats are as high as those from fasted rats.

5.3.5 A Note on the Final Perfusate Sample

Reference has been made occasionally to anomalous concentrations of metabolites in the 75 minute perfusate sample. Ammonia sometimes showed a rise during the last 15 minutes of perfusion (Chapter 3), as did glutamine in hearts perfused with it (Chapter 5, Section 5.2.5.1), whereas aspartate in two aspartate-perfused hearts showed a slight drop in concentration. These changes did not occur in all hearts, and might be attributed to incipient heart failure. However, some hearts were maintained for longer periods without any continued increase in the perfusate ammonia concentration, and hearts which showed a rise in ammonia concentration in the last 15 minutes included hearts perfused for 60, 75, 90 or 120 minutes. Attempts to trace an artefact connected with the taking of the final sample were unsuccessful. In most cases the increases were small and within the limit of experimental error. An uptake of aspartate in the final 15 minutes was not supported by any significant increase in alanine or ammonia production.

CHAPTER 6

THE LOCALIZATION IN RAT HEART OF SOME ENZYMES RELATED TO GLUTAMATE METABOLISM

Chapter 6. The Localization in Rat Heart of Some Enzymes Related To Glutamate Metabolism

There are two major approaches to the intracellular localization of enzymes in a tissue. The first involves physical disruption of the tissue and the separation of subcellular organelles by appropriate methods, for example, differential centrifugation; the enzymes are measured in the resultant fractions by standard techniques. This approach is limited by the number of fractions which can be separated: usually only nuclei, mitochondria and microsomes are segregated from the soluble components of the cytoplasm. The microsome fraction is itself heterogeneous, containing ribosomes, endoplasmic reticulum and other membranes, fragments of which may roll up in such a way as to enclose enzymes which are actually cytoplasmic. Cross-contamination between fractions is a further disadvantage of this method, but can be reduced by employing gentler methods. One of these is the fractional extraction technique of Pette and co-workers (Pette, 1966, 1968). This method permits gentle but efficient washing-out of cytoplasmic enzymes so that it is no longer so necessary to depend on the measurement of compartment-specific "marker" enzymes. The standardization of all steps in the procedure leads to excellent reproducibility. The laboratory method has been described (Chapter 2, Section 2.4.2.1). Fractional extraction has been successfully applied to a wide range of problems (Pette et al., 1962a,b; Pette and Luh, 1962; Klingenberg and Pette, 1962; Pette, 1966; Goebell and Pette, 1967; Pette, 1968; Brdiczka et al., 1969; Brdiczka and Pette, 1971). Its particular disadvantage is that the centrifugation steps precipitate nuclei and other

subcellular organelles in addition to mitochondria, all of which can be seen microscopically to be intact after the first sucrose wash (Pette, 1968). Hence homogenization of the suspended pellet yields a supernatant (S4) containing nuclear and microsomal, as well as mitochondrial enzymes. All membrane-bound enzymes appear in the final pellet, whether they are attached to the inner mitochondrial membrane (as is SDH) or to the outer membrane (as is monoamine oxidase) or to other membranes such as the endoplasmic reticulum.

The second approach permits the direct observation of enzyme distribution in situ without tissue disruption, achieved by the histochemical staining of the enzyme in a thin section of rapidly-frozen tissue. A reaction catalyzed by the enzyme under investigation results in the deposition of a detectible product at the site of the enzyme. The product is coloured in light-microscope work, or electron-opaque for electron microsocpy. Tetrazolium salts have been widely used in the light-microscopic study of enzymes in tissue slices; the staining reaction involves the reduction of a soluble, pale yellow tetrazolium salt to a densely-coloured formazan. Ditetrazolium salts yield dark blue insoluble formazans and are used in preference to monotetrazolium salts which yield more readily-diffusible red formazans. Nitro-blue tetrazolium is most widely used as it is easily reduced in the standard technique, without significant non-specific reduction (known as the "nothing dehydrogenase" reaction), and produces a small-grained formazan suitable for accurate enzyme localization. In dehydrogenase localization reducing equivalents are provided (via a reduced coenzyme) by the substrate of the dehydrogenase. Other enzymes can be localized if they can be

coupled to a suitable dehydrogenase.

Tetrazolium salts react directly with reduced flavoproteins. Thus the first enzymes to be studied by tetrazolium staining were succinate dehydrogenase and the flavoprotein diaphorases (Seligman and Rutenberg, 1951; Farber and Louviere, 1956; Farber and Bueding, 1956; Farber et al., 1956; Nachlas et al., 1957). Diffusion of these enzymes during the staining period was not a problem in their localization, since they are membrane-bound. NADH does not reduce tetrazolium at an appreciable rate, so that the localization of an NAD^+-linked dehydrogenase requires an electron-transfer intermediate between NADH and tetrazolium. In earlier experiments endogenous tetrazolium reductases fulfilled this function, with the disadvantage that their distribution, as well as that of the enzyme under investigation, was reflected in the formazan pattern obtained. This problem was overcome in later work by the incorporation of soluble redox dyes in the staining medium (Farber and Bueding, 1956; Nachlas et al., 1957); phenazine methosulphate is generally used. A further consequence of the use of PMS was a reduction in staining time which allowed the localization of soluble dehydrogenases present in sufficient activity without much diffusion of the enzymes during staining. However, soluble enzymes tended to diffuse out of the tissue with the result that much of the formazan was deposited in the liquid medium, staining in the tissue slice being weak and diffuse. Leakage of enzyme was greatly reduced by incorporating the staining reagents in a slice of gelatine (Fahimi and Amarasingham, 1964) or of agarose gel (Pette and Brandau, 1962) which was applied to the tissue slice. An agarose gel staining system has been used successfully in the localization of dehydrogenases and other enzymes

in several tissues (Pette and Brandau, 1962, 1966; Brandau and Pette, 1966; Sigel and Pette, 1969). Histochemical localization in heart muscle, however, has been confined to structure-bound enzymes such as succinate dehydrogenase, glycerophosphate oxidase and adenosine triphosphatase (Niles et al., 1964; Morales and Fine, 1965; Chayen et al., 1966; Niles et al., 1966; Pette and Brandau, 1966).

The patterns produced by staining skeletal muscle are very clear. Mitochondrial enzymes such as succinate and glutamate dehydrogenases cause formazan deposition only in the mitochondria opposite the I-bands (Brandau and Pette, 1966). The I-bands of adjacent fibrils coincide in the muscle studied by these authors, resulting in a striated pattern in which the narrow stained bands are composed of individual mitochondria. Lactate dehydrogenase is an extra-mitochondrial enzyme, but it appears not to be homogeneously distributed throughout the system since formazan is deposited in the I-bands of the fibrils. This gives a striated distribution in which the stained bands are wider than those of mitochondrial enzymes, and in which individual mitochondria cannot be seen (Brandau and Pette, 1966). It is inferred that the enzyme occurs only in the I-bands of the fibrils. Malic enzyme catalyzes formazan deposition between and parallel to the fibrils, suggesting that it is located in the interfibrillar spaces. Enzymes found in two compartments give a composite formazan distribution, for example, glyceraldehyde phosphate dehydrogenase stains heavily in the I-band mitochondria; this pattern is superimposed on a higher staining of the I-band cytoplasm. Malate dehydrogenase stains lightly in the interfibrillar spaces and heavily in the interfibrillar mitochondria (Brandau and Pette, 1966).

Skeletal muscle contracts when removed from its attachment in the animal, and only shows striations if it is frozen while held in a stretched position. Since the muscle fibrils run parallel to the axis of the muscle, striated fibrils will be seen in a longitudinal section of a muscle prepared in this manner. Heart muscle differs in that the orientation of the fibres does not conform to any regular pattern; sections cut from it generally show a mixture of fibres cut in transverse, longitudinal and oblique section. Further, it is technically difficult to stretch heart muscle during freezing. In the present investigation, some relaxation was induced by soaking small pieces of heart muscle in isotonic KCl solution before freezing, and striations were visible in such preparations. Heart muscle also differs from skeletal muscle in its cellular organization. The fibres are shorter, so that coincidence between the I-bands of adjacent fibrils is unlikely to persist beyond a few sarcomere-lengths. The mitochondria are larger, and have a more complex structure than those of skeletal muscle and they practically fill the interfibrillar spaces (Klingenberg, 1964).

In the present chapter the activity and intracellular distribution in heart muscle of three key enzymes concerned in nitrogen metabolism, GluDH, GOT and GPT, are investigated and discussed. The methods used are tetrazolium staining (Brandau and Pette, 1966) and fractional extraction (Pette, 1968). The distributionsof the enzymes under study are compared with those of marker enzymes. Control experiments are described in an attempt to assess the contribution to the stain distribution of artifactual adsorption of enzymes to histological structures. Reports of this work have already appeared (Jarvie

Figs. 6.1 - 6.6 Distribution of enzymes in sections of rat cardiac muscle stained as described in the text. Staining times (in parenthesis) and magnifications are given.

Fig. 6.1 Lactate dehydrogenase, tetrazolium method (10 min) x 1100.

Fig. 6.2 Succinate dehydrogenase, tetrazolium method (10 min) x 1100.

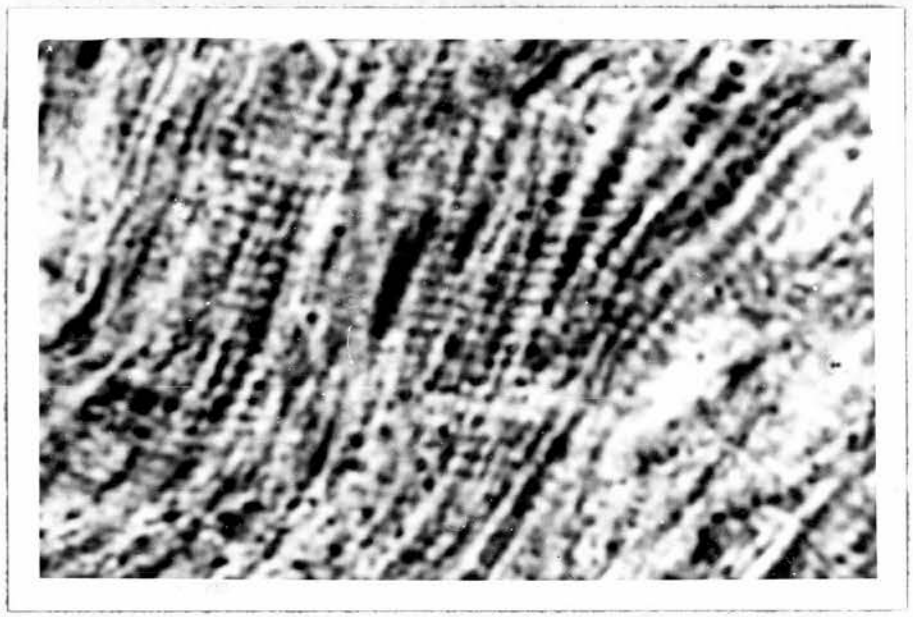
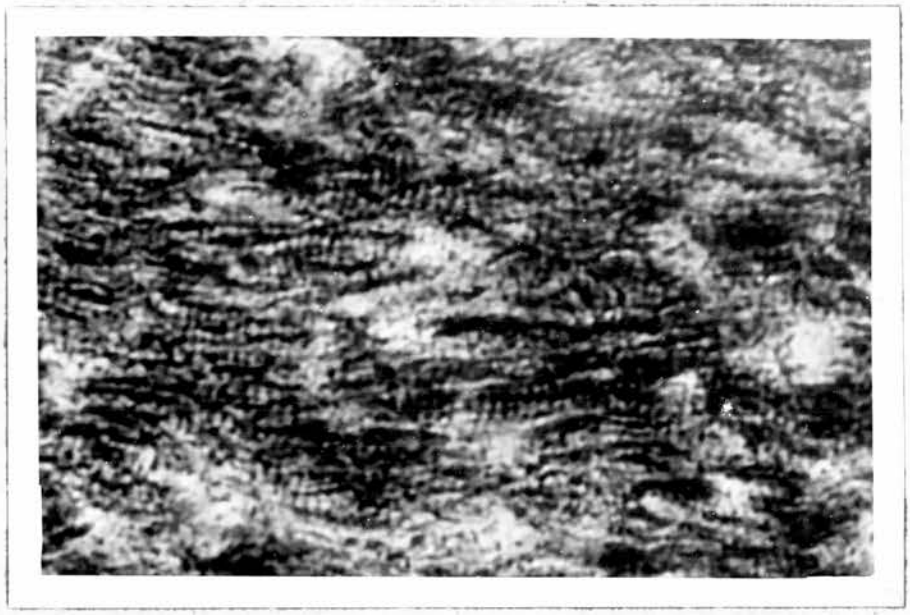


Fig. 6.3 Succinate dehydrogenase, tetrazolium method (10 min) x 450.

Fig. 6.4 Cytochrome oxidase, diaminobenzidine technique (40 min) x 450.



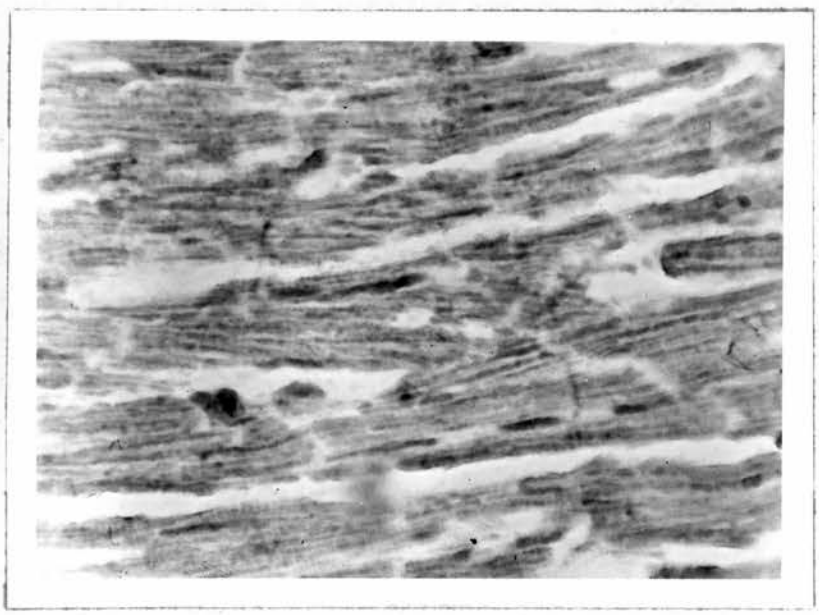
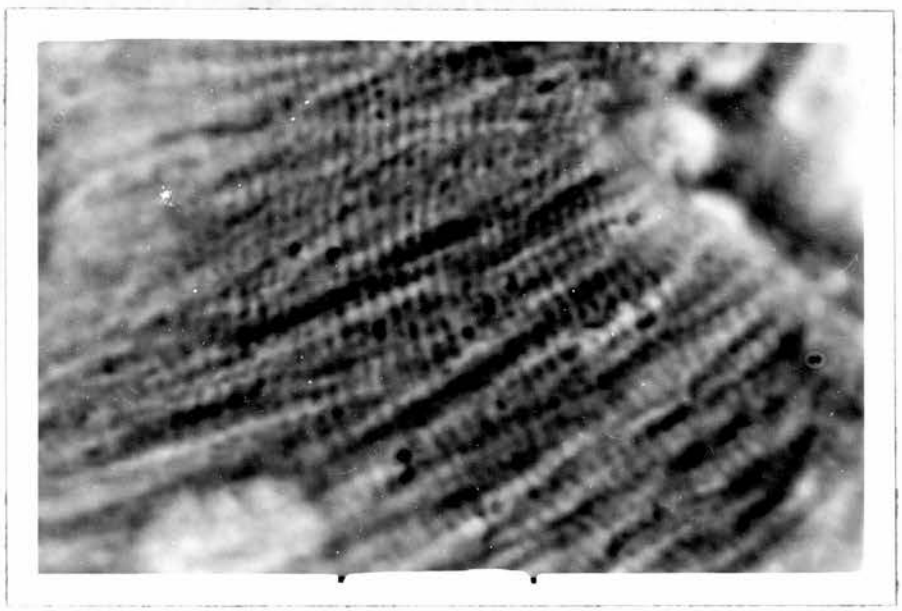


Fig. 6.5 Glutamate dehydrogenase, tetrazolium method (30 min) x 1100.

Fig. 6.6 Glutamate oxaloacetate transaminase, tetrazolium method (30 min), x 1100.



(a)

Enzyme	Figure	L (μm)	R
LDH	1	2.3	0.81
GluDH	5	2.3	0.86
GOT	6	2.3	0.78
ADH, added	7	2.3	0.91
Cytochrome oxidase	4	2.3	0.98
SDH	2	4.3	1.32
SDH	3	4.1	1.32

(b) Literature values (for SDH)

R	Reference
0.86	Chayen et al., 1966
1.39	Burstone, 1962
1.40	Nachlas et al., 1957
1.43	Nachlas et al., 1957
1.54	Novikoff, 1959

Table 6.1 Dimensions of banding patterns in heart myofibrils stained by the techniques described.

L is the distance between successive dark striations; R is the ratio of L divided by the distance between adjacent fibrils. L was obtained by measuring 10 consecutive striations along a fibril and dividing by 10; this procedure was repeated at least 5 times for each photograph and the mean was calculated. The mean distance between fibrils was obtained in an analogous manner. Literature values were obtained by making similar measurements on published photographs. For these only the ratio is reliable, since in many cases the actual magnification of the published picture is uncertain.

et al., 1971; Jarvis and Ottaway, 1975).

6.1 Histochemical Localization of Enzymes in Rat Heart

Definition: In the present chapter the phrase "soluble enzymes" denotes enzymes which are water-soluble and free to diffuse in the aqueous medium created on disruption of the tissue. Such enzymes may or may not diffuse freely within the cellular environment, and the phrase covers cytoplasmic and mitochondrial enzymes. It also includes enzymes which may be loosely adsorbed in vivo to structures such as fibrils, but which are released by standard extraction methods. "Insoluble enzymes" denotes enzymes firmly bound to membranes, from which they can only be released by special solubilization techniques. Examples are SDH and monoamine oxidase. A list of enzyme abbreviations can be found in Chapter 2, Section 2.1.3.

Thin sections were cut from rapidly-frozen rat heart as described in Chapter 2 (Section 2.4.1.1). In most sections, phase-contrast microscopy revealed areas of transversely cut fibrils and areas of longitudinally cut fibrils. Some longitudinally cut fibrils, but not all, showed striations visible in polarized light. The potassium chloride treatment thus relaxed many of the fibres, but its effect on the rest of the fibres was limited by the extent of diffusion of K^+ ions through the tissue. All striated fibres, however, were completely relaxed, and showed a sarcomere-length of 2.3 μm (see Table 6.1).

The stain distributions given by five enzymes in sections of rat heart were photographed (Figs 6.1 - 6.6). The contrast is not so

sharp as in the photographs of Brandau and Pette (1966) or Sigel and Pette (1969), possibly because longer staining times were required for heart muscle enzymes (other than SDH and LDH) than those recommended for skeletal muscle enzymes (Sigel and Pette, 1969), so that some diffusion of enzyme or of reaction intermediates may have occurred during the staining period. Control sections incubated in the absence of substrate did not stain to any extent: there was no "nothing dehydrogenase" reaction.

6.1.1 Lactate Dehydrogenase

The staining pattern of LDH is characterised by formazan deposition in the fibrils (running from bottom to top, Fig 6.1); some fibrils are more heavily stained than others. The fibrillar staining is crossed by striations (left to right, Fig 6.1) which are "in register" for about 35 fibril widths in the section photographed. Whereas the striations observed with skeletal muscle LDH are continuous across adjacent fibrils, giving the picture a "banded" appearance (Brandau and Pette, 1966), those of LDH in heart muscle are broken so as to give a "blotched" appearance. This may be due to the presence of bulky unstained mitochondria between the fibrils of heart muscle (Klingenberg, 1964). Calculation of the distance along the fibril between successive striations gives a value of 2.3 μm (Table 6.1), which is approximately equal to the quoted sarcomere-length of 2.5 μm (Bloom and Fawcett, 1968). The striations probably correspond to a concentration of the enzyme in the I-band cytoplasm. Calculation of the sarcomere-length from the photographs requires accurate knowledge of the absolute magnification. This is

not necessary if the length is measured in terms of a quantity which is assumed to be constant in all photographs. Hence the ratio R given in Table 6.1 is the mean distance between striations divided by the mean distance between fibrils in each photograph.

6.1.2 Succinate Dehydrogenase and Cytochrome Oxidase

The pattern given by SDH also shows striations (Figs 6.2 and 6.3), but these are much coarser in appearance than those given by lactate dehydrogenase. (Figs 6.1 and 6.2 are at the same magnification; Fig 6.3 shows staining of SDH at rather less than half of the magnification of Fig 6.2). Fig 6.3 shows parallel bands of stain running from left to right which may represent staining of interfibrillar mitochondria. The striations superimposed upon them (running from bottom to top) are not "in register" for more than 5 - 6 fibril widths (compare LDH), and the distance between adjacent striations is 4.2 μm (Table 6.1). This distance is rather shorter than two sarcomeres, and the basis of the periodicity is not known. The pattern resembled that observed by Chayen et al. (1966), but lacked the granular texture described by Niles et al. (1966) in the presence of PMS.

Cytochrome oxidase, like SDH, is bound to the inner mitochondrial membrane. It was localized by diaminobenzidine staining (Chapter 2), in an attempt to eliminate any possible artifacts of the tetrazolium technique. The resultant pattern (Fig 6.4) can be compared with figure 6.3, which was taken at the same magnification. The bands of stain running from left to right probably represent interfibrillar mitochondria. With a few exceptions, there are no

striations, and where striations occur, they are weak and show a periodicity corresponding to the sarcomere-length (Table 6.1). The staining time was necessarily much longer (40 minutes) than that required for the staining of SDH by the formazan technique, and the stain deposition is weaker and more diffuse.

6.1.3 Glutamate Dehydrogenase

GluDH is generally regarded to be a mitochondrial enzyme (de Duve et al., 1962). This is supported by fractional extraction (see below). The stain ought therefore to be deposited in and around the interfibrillar mitochondria. However, the staining pattern observed (Fig 6.5) is superficially similar to that of lactate dehydrogenase (Fig 6.1). Fibrils run from left to right, and striations cross them at right angles and are in register for many fibril widths. Dark spots of heavy formazan deposition are also visible. These are unlikely to be fibrils cut in cross-section, since some of them occur singly in the area of longitudinal section. They may represent the position of structures rich in GluDH, possibly discrete mitochondria or larger T-tubules.

6.1.4 Glutamate Oxaloacetate Transaminase

GOT (Fig 6.6) yields a similar pattern to that of GluDH. The striated fibrils run from left to right, and a few dark spots are visible. The greyness of the picture is due to the thickness of the section. In both GOT and GluDH patterns diffuse bands of stain run parallel to the fibrils, which may represent an underlying formazan deposition in interfibrillar mitochondria. They are not

shown by lactate dehydrogenase.

6.1.5 Control Tests

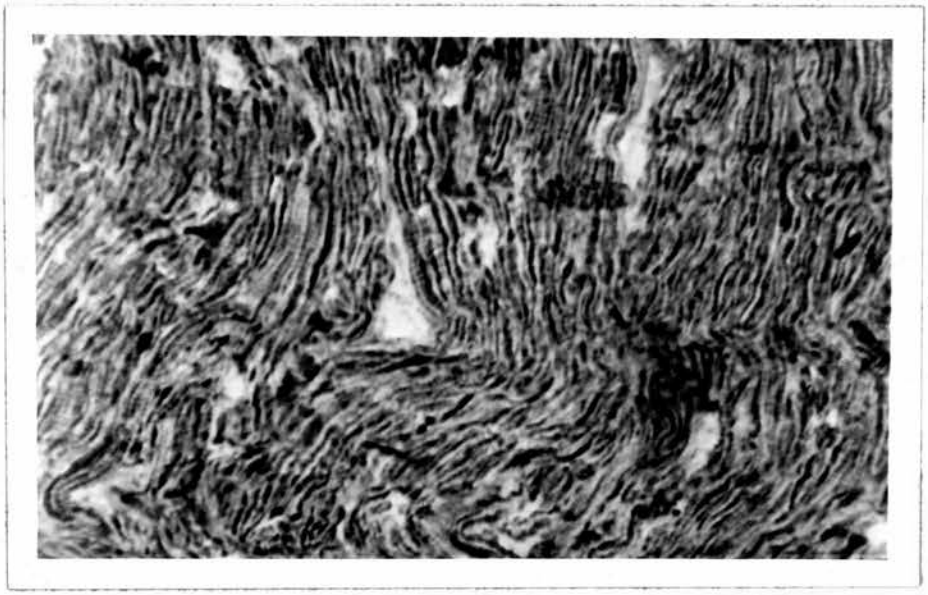
The similarity of the staining pattern of soluble intramitochondrial enzymes to that of LDH raises the possibility that these enzymes leak from mitochondria ruptured in the freezing and thawing of the tissue, and selectively adsorb to fibrillar elements in the cytoplasm. It has been shown that aldolase and other glycolytic enzymes bind to muscle proteins, particularly to actin (Arnold and Pette, 1968, 1970; Arnold et al., 1969, 1971). This affinity may be responsible for the I-band distribution of soluble enzymes stained in muscle slices. It is therefore important to test the adsorption of enzymes to heart muscle fibres, both in vitro in a heart homogenate and microscopically in a tissue slice. An enzyme not naturally present in rat heart was used in these tests in order to avoid interference by endogenous enzyme.

Alcohol dehydrogenase is absent from rat heart (Morales and Fine, 1965), but can be stained by the tetrazolium technique in muscles which possess it (de Pereda and Rebollo, 1967), and is therefore an appropriate enzyme to test for adsorption to heart myofibrils. A solution of yeast ADH was applied to tissue slices and stained as described in Chapter 2 (Section 2.4.1.6). The resultant pattern (Fig 6.7) showed that selective adsorption of the enzyme to elements of the muscle tissue had indeed occurred. The stain distribution resembles that of SDH (Fig 6.3) rather more closely than that of LDH (Fig 6.1), suggesting that the enzyme is taken up by the interfibrillar spaces, although the abundant striations

Figs. 6.7 and 6.8 Control experiments to test for adsorption of components of the staining system to elements of the tissue slice. Magnification x 450.

Fig. 6.7 The formazan distribution obtained on staining a tissue, previously suffused with alcohol dehydrogenase, with a staining gel for localization of this enzyme (5 min). Some striations are visible, suggestive of fibrillar adsorption, but the predominant dark bands suggest that much of the enzyme assumes a location in the interfibrillar spaces.

Fig. 6.8 The pattern obtained on staining a tissue previously suffused with NADH solution (10 min). There are no formazan granules or striations in the tissue: all the formazan is deposited in the gel-slice. Thus adsorption of NADH or other components of the staining system can be excluded.



		ADH activity extracted (μmol/min at 25°C)	% of total activity added
Fractions	S_1	7.27	86.5
	S_2	1.19	14.2
	S_3	0.00	0.0
	P	0.00	0.0
Total activity recovered		8.46	-
Recovery (%)		-	100.7

Table 6.2 Adsorption of yeast ADH to a rat heart homogenate.

0.5 g rat heart was homogenized in 10 ml of sucrose medium (Chapter 2, Section 2.4.2.2). 8.40 units of ADH (1 unit = 1 μmol of ethanol per min at 25°C) were added and the mixture was stirred gently at 0°C for 20 min. Centrifugation yielded supernatant S_1 and a pellet, which was resuspended in sucrose medium and stirred for 15 min. The resultant supernatant was S_2. A subsequent 15 min wash of the pellet with 0.1 mol/l phosphate buffer, pH 7.2, yielded supernatant S_3 and the pellet P.

have a periodicity corresponding to the sarcomere-length (Table 6.1). The distribution was unaffected by the ionic strength of the solution in which the enzyme was dissolved and applied.

If NADH solution was applied to the tissue slice in analogous manner and stained with a gel containing Nitro-BT and PMS, all the formazan was precipitated in the staining gel, and none was deposited in the tissue (Fig 6.8). This result shows that there is no binding of NADH or other components of the staining system to the tissue.

In vitro adsorption of alcohol dehydrogenase to elements of a heart muscle homogenate was also tested (Chapter 2, Section 2.4.2.2). However, all the enzyme activity added to such homogenates could be extracted by a medium of low ionic strength (Table 6.2), indicating that no enzyme is adsorbed to the disrupted tissue under these conditions.

6.2 Enzyme Localization by Fractional Extraction

The result of the fractional extraction of enzymes in a rat heart by the method of Pette (1968) (Chapter 2, Section 2.4.2.1) is shown in Table 6.3 and Fig 6.9. Three enzymes concerned with nitrogen metabolism, GluDH, GOT and GPT, are compared with marker enzymes having four types of distribution: soluble-cytoplasmic (LDH, pyruvate kinase, adenylate kinase); soluble-intramitochondrial (citrate synthetase); mixed (malate dehydrogenase); and mitochondrial membrane-bound (SDH).

Glutamate pyruvate transaminase is present in low activity, and its measurement is thus less accurate than that of the marker

Legend to Tables 6.3 and 6.4

0.5 g of tissue was analyzed by fractional extraction as described in Chapter 2, Section 2.4.2.1. Fractions were obtained as follows. S_1, 15 min gentle stirring of suspension in sucrose medium, low ionic strength. S_2, 15 min repeat of this procedure. S_3, 15 min gentle stirring in 0.1 mol/l phosphate. S_4, homogenization followed by 10 min stirring. P, resuspended pellet.

Enzyme	Total activity, μmol/min/g fresh weight at 25°C	Percentage of total activity in each fraction					Cumulative percentage of total activity extracted				
		S_1	S_2	S_3	S_4	P	S_1	S_2	S_3	S_4	P
Pyruvate kinase	69.9	93.8	2.5	0.7	2.2	1.1	93.8	96.3	97.0	99,2	100.0
Adenylate kinase	193.5	86.9	3.6	5.7	3.6	0.3	86.9	90.5	96.2	99.8	100.0
Lactate dehydrogenase	478.4	96.7	1.6	0.4	1.2	0.2	96.7	98.3	98.7	99.9	100.0
Glutamate pyruvate transaminase	0.78	68.7	31.4	0.0	0.0	0.0	68.7	100.0	100.0	100.0	100.0
Malate dehydrogenase	1309.5	33.9	3.0	6.1	52.7	4.4	33.9	36.9	43.0	95.7	100.0
Glutamate oxaloacetate transaminase	196.8	25.0	3.2	3.2	62.4	6.1	25.0	28.2	31.4	93.8	100.0
Citrate synthetase	56.6	4.9	0.3	4.5	77.8	12.6	4.9	5.2	9.7	87.5	100.0
Glutamate dehydrogenase	12.9	5.0	2.5	5.0	75.7	12.0	5.0	7.5	12.5	88.2	100.0
Succinate dehydrogenase	26.7	0.0	0.0	0.1	1.1	98.9	0.0	0.0	0.1	1.1	100.0
Protein	118.5 mg/g	23.4	3.0	3.5	12.5	57.4	23.4	26.5	30.0	42.5	100.0

Table 6.3 Fractional extraction of enzymes in rat heart.

Enzyme	Total activity, μmol/min/g fresh weight at 25°C	Cumulative percentage of total activity extracted				
		S_1	S_2	S_3	S_4	P
Lactate dehydrogenase	602.9	89.4	93.2	98.4	99.5	100.0
Glutamate pyruvate transaminase	12.0	90.3	99.8	100.0	100.0	100.0
Malate dehydrogenase	534.9	41.1	46.7	49.6	94.1	100.0
Glutamate oxaloacetate transaminase	91.6	14.0	16.3	19.7	89.5	100.0
Citrate synthetase	9.9	1.1	2.0	8.7	90.1	100.0
Glutamate dehydrogenase	221.4	0.7	1.0	3.5	86.8	100.0
Succinate dehydrogenase	16.8	0.0	0.0	0.0	0.6	100.0
Protein	164.2 mg/g	48.0	52.1	56.4	78.3	100.0

Table 6.4 Fractional extraction of enzymes in rat liver.

Figs. 6.9 and 6.10 Fractional extraction of enzymes in tissue.

0.5g of rat tissue was analyzed by the fractional extraction technique (see Chapter 2, Section 2.4.2.1). Fractions were obtained as follows: S_1, 15min gentle stirring of suspension in sucrose medium, low ionic strength; S_2, 15min repeat of this procedure; S_3, 15 min gentle stirring in 0.1 mol/l phosphate; S_4, homogenization followed by 10 min stirring; P, resuspended pellet.

Enzymes are labelled as follows:

a lactate dehydrogenase

b pyruvate kinase

c adenylate kinase

d glutamate pyruvate transaminase

e malate dehydrogenase

f glutamate oxaloacetate transaminase

g glutamate dehydrogenase

h citrate synthetase

i succinate dehydrogenase.

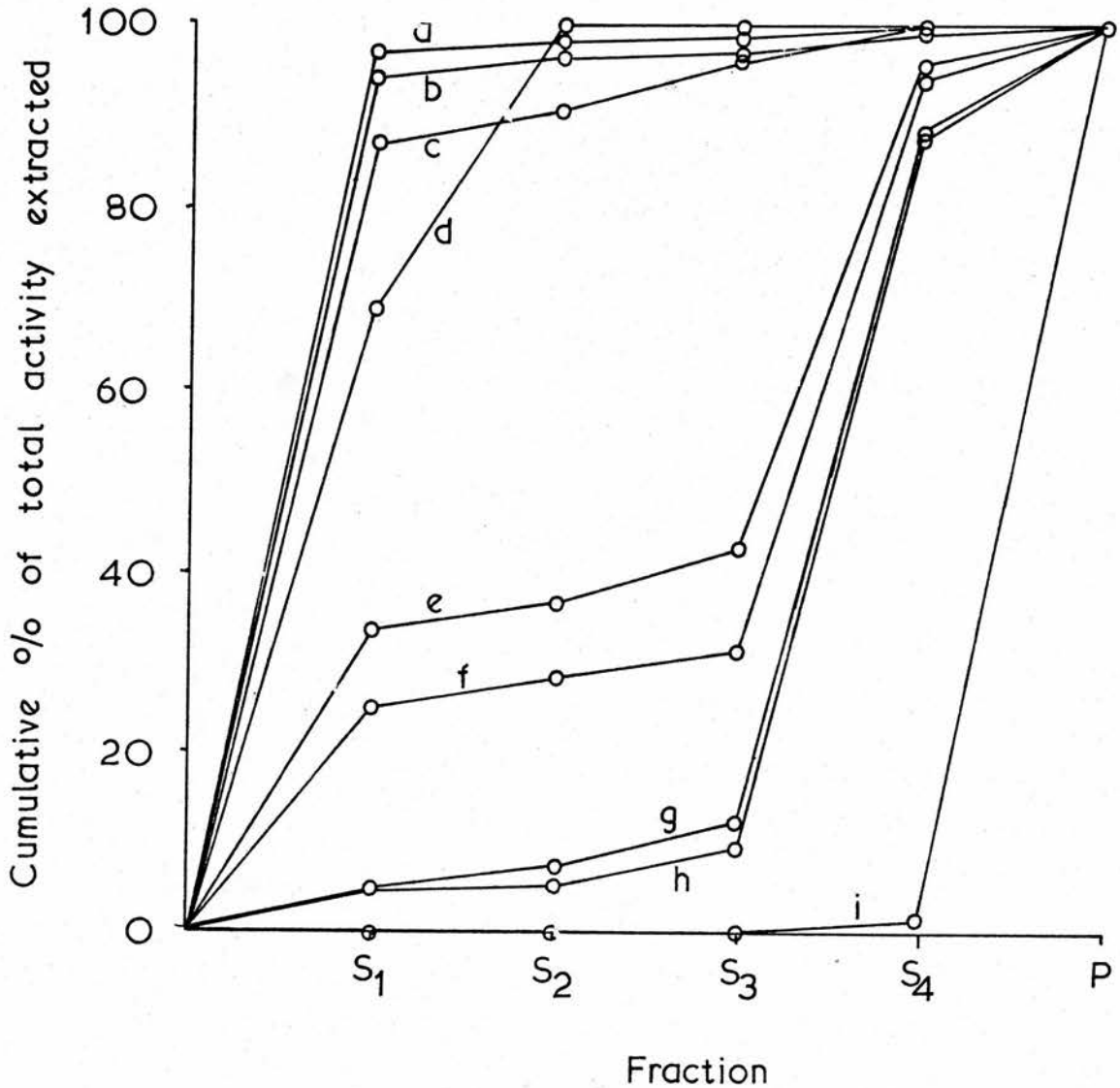


Fig. 6.9 Fractional extraction of enzymes in rat heart.

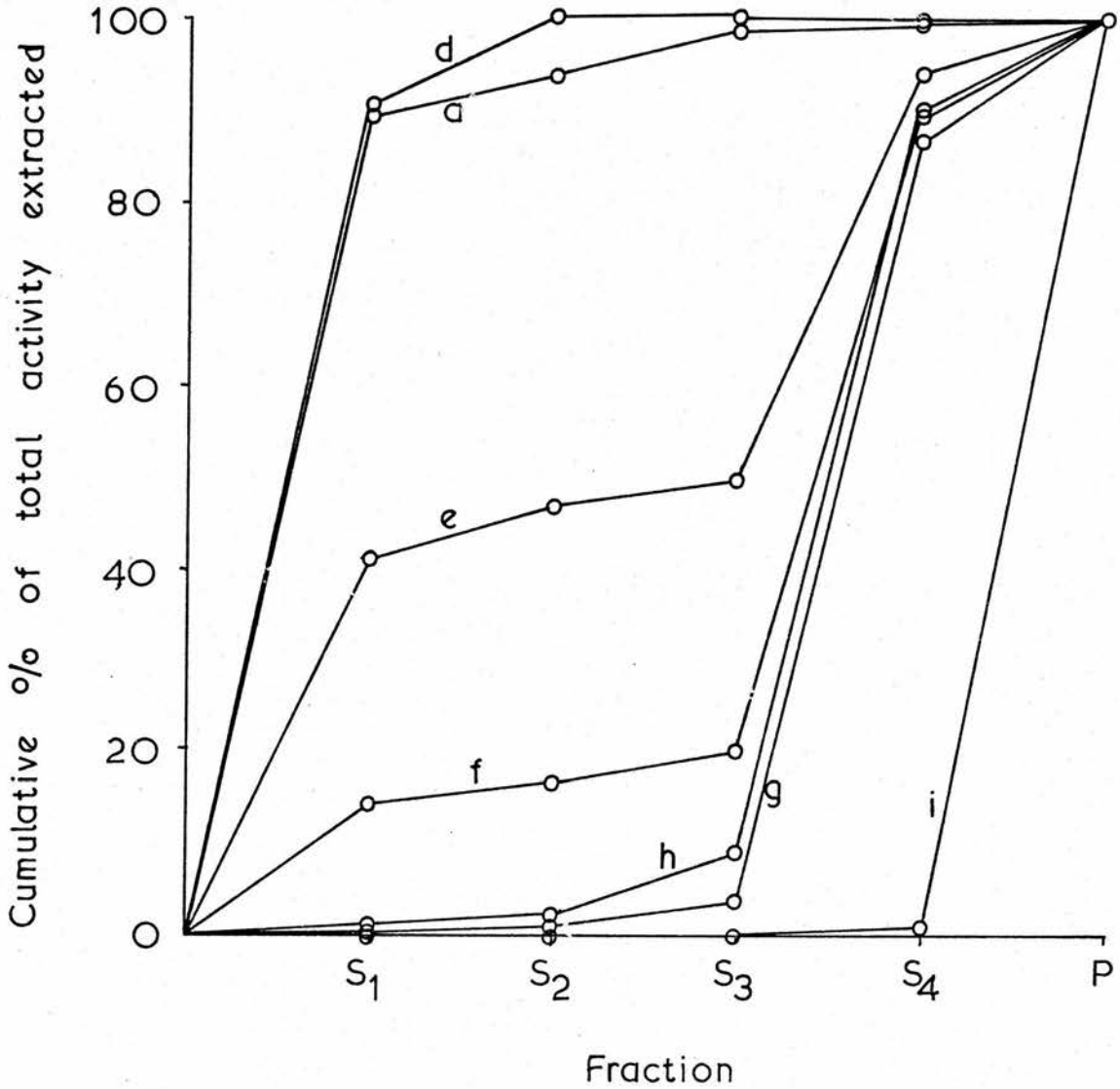


Fig. 6.10 Fractional extraction of enzymes in rat liver.

enzymes. The fractional extraction pattern, however, shows that this enzyme is entirely cytoplasmic. GOT in heart is more active than either GPT or GluDH, and can be separated into two fractions: soluble-cytoplasmic and soluble-intramitochondrial. Its distribution resembles that of malate dehydrogenase, although the proportion of cytoplasmic to mitochondrial is lower in the case of GOT. GluDH is more active in heart than it is in skeletal muscle (Lowenstein, 1972), and its distribution is that of a soluble-intramitochondrial enzyme such as citrate synthetase.

In the fractional extraction of rat liver or rabbit muscle (Pette, 1968), no more than 5% of the total activity of soluble-intramitochondrial enzymes is released in the washing stages prior to homogenization. In the present investigation on the other hand, 12.5% of the total activity of GluDH is liberated prior to homogenization. The parallel release of citrate synthetase suggests that some of the mitochondria are ruptured during the sucrose and phosphate washes. These data thus provide no evidence of any extramitochondrial glutamate dehydrogenase. As a control of the technique, a fractional extraction of rat liver was carried out in which less than 4% of the GluDH was released prior to homogenization (Table 6.4 and Fig 6.10), comparable with the results of Pette (1968).

6.3 Discussion

The photographs comprising Figs 6.1 - 6.6 show that the enzymes studied conform to specific distributions throughout rat cardiac muscle, as they do in skeletal muscle. The more diffuse nature of formazan deposition in cardiac muscle makes it rather difficult to

deduce with certainty to which cellular structures the staining corresponds, whereas the clear staining pattern given by skeletal muscle allows much greater precision in allocating formazan deposition to fibrils, I-bands, mitochondria or interfibrillar spaces (Brandau and Pette, 1966; Sigel and Pette, 1969). In heart muscle it is often hard to decide whether bands of stain represent fibrils or the interfibrillar spaces running parallel to the fibrils. The presence of striations with a periodicity of one sarcomere-length, dividing the band of stain into a row of dark spots, suggests fibrillar staining. The soluble enzymes showed such a pattern, and the dark spots presumably indicate a concentration of enzyme in the I-bands. More continuous bands of stain can be seen in the SDH and cytochrome oxidase distributions (Figs 6.3 and 6.4); similar though fainter bands underly the GluDH and GOT distributions (Figs 6.5 and 6.6). These may represent interfibrillar mitochondria. Exogenous ADH (Fig 6.7) gives similar bands though they are thicker and more distinct, corresponding to interfibrillar spaces which have soaked up the added enzyme.

In view of this difficulty in relating the histochemical pattern of heart tissue to known cellular structures, it is more useful to compare the patterns given by the different enzymes, and to relate these to the distributions of the enzymes revealed by tissue fractionation. The most striking feature of figures 6.1 - 6.6 is the similarity in the formazan distribution given by all the soluble enzymes, irrespective of their extra- or intramitochondrial location as shown by the extraction technique (Fig 6.9). The similarity in appearance is reflected by the similarity of the tissue dimensions: the distance between striations is equal to the sarcomere

length (2.3 μm) and the ratio R (Table 6.1) is the same in all cases. Membrane-bound enzymes alone gave a different pattern. The distance between SDH striations was 4.2 μm, and R was correspondingly greater. The basis of the 4.2 μm periodicity, presumably a mitochondrial feature, is unknown. Comparison with cytochrome oxidase stained with diaminobenzidine (Fig 6.4) suggests that an artifact of the tetrazolium system may be involved.

Although an I-band location of heart LDH and cytoplasmic GOT is feasible and is analogous to the distribution of LDH and many glycolytic enzymes in skeletal muscle (Brandau and Pette, 1966; Sigel and Pette, 1969), the similar distribution of GluDH is unexpected. Apart from the faint and rather ambiguous bands already pointed out, neither GluDH nor GOT were associated with significant formazan deposition in mitochondria. The possibility was considered that the GluDH stained in the cytoplasm originated from mitochondria ruptured in the freezing and thawing process, and became adsorbed to actin in the myofibrils. This hypothesis was supported by the finding that exogenous ADH added to the tissue assumed a striated distribution. However, the pattern obtained with ADH was more like that of SDH than that of the soluble enzymes, and a large amount of stain was deposited in the interfibrillar spaces: this was not seen with either GluDH or GOT. Furthermore, adsorption of ADH to actin did not occur in vitro in a heart muscle homogenate.

The compartmentation of ammonia metabolism in heart has already been discussed (Chapters 3 and 4). The small and metabolically active exchangeable ammonia pool would be associated with a separate fraction of glutamate dehydrogenase. Location of this fraction in the sarcotubular system would be in agreement with the observed

findings. It would be separated from the rest of the cytoplasm by a membrane, maintaining physical separation of the ammonia pools. It would be easily accessible to substrates such as external ammonia, hence the speed at which added ammonia appears to be metabolized by a small pool of enzyme. It would stain as a fibrillar enzyme; being more accessible than mitochondrial GluDH to exogenous substrate, it might well stain whilst the latter remained unstained. (Tager (1966) suggested that mitochondrial GluDH might be $NADP^+$-specific, a further possible reason for non-staining of the mitochondrial enzyme by an NAD^+-containing staining medium). Finally, it would appear in fractional extraction as a mitochondrial enzyme, since the tubular membrane would be unbroken until the final homogenization step which opens the mitochondria. In addition, factors such as the small size of the enzyme pool or special lability of the enzyme might have prevented its previous discovery.

CHAPTER 7

GENERAL DISCUSSION AND CONCLUSIONS

Chapter 7 General Discussion and Conclusions

The study presented here has several aspects. The ammonia concentration has been measured in rat heart and is similar to concentrations reported both in skeletal and in cardiac muscle by other authors. It has been shown that rat heart produces ammonia during perfusion with media to which none has been added, and conversely that ammonia added to the perfusing medium can be taken up and metabolized by the heart. A distribution ratio is maintained by the perfused heart, in which the concentration in the tissue can be up to twenty times as high as that in the medium. The effect on this ratio of varying the perfusate ammonia concentration has been described. Transport of ammonia across the plasma membrane has been investigated by measuring the ammonia distribution under different conditions and also by the use of isotopically-labelled ammonia. The relationship of ammonia metabolism to that of free amino acids in heart has been investigated, and the localization of some of the enzymes involved in this metabolism has been studied.

7.1 Source of Ammonia

In skeletal muscle, the production of ammonia is well-documented (see Chapter 1) and derives from the breakdown of adenine nucleotides (Lowenstein, 1972). For the reasons discussed earlier (Chapter 5, Section 5.1), however, adenine nucleotides cannot be a significant source of ammonia in rat heart, and one is led to presume that ammonia is formed in this tissue from the intracellular pool of free amino acids. Both glutamate and glutamine can give rise to ammonia by simple hydrolytic reactions: both enzymes are present in rat

heart (glutamate dehydrogenase, Lowenstein, 1972; glutaminase, Ottaway, 1969a,b). At first sight glutamate is the more likely precursor: on perfusion a decrease in tissue glutamate occurs which is equal to the amount of ammonia produced (Chapter 5, Section 5.2.3.3). The liberation of ammonia from glutamate is a final step in the deamination of all amino acids which can transaminate with α-oxoglutarate, allowing their carbon skeletons to be used in energy metabolism. However, hearts perfused with added glutamate took up glutamate from the medium but did not produce significantly more ammonia although the tissue ammonia concentration was significantly raised. (These hearts did, however, produce more glutamine than those perfused without a nitrogen source. The net reaction 2 moles glutamate $\longrightarrow$ 1 mole glutamine + 1 mole α-oxoglutarate could occur without any increase in net ammonia production. This pathway requires glutamine synthetase and is discussed further in the following section). Finally, the equilibrium of glutamate dehydrogenase in rat heart mitochondria (Chapter 1, Section 1.1) suggests that the enzyme functions in the synthesis rather than in the degradation of glutamate. If the enzyme is to catalyze the release of ammonia from glutamate, compartmentation of the enzyme substrates would be required, for example a high concentration of glutamate at the site of the enzyme, or a mechanism whereby the relatively high concentration of ammonia in the tissue is prevented from reaching the enzyme.

In contrast, perfusion of hearts with added glutamine did lead to a significant increase in ammonia production, although hearts perfused without an added nitrogen source produced ammonia without any detectable disappearance of glutamine. The metabolism of ^{15}N-ammonia by the heart (Chapter 4) is in accordance with ammonia

production from glutamine, with kinetic constants as determined by Ottaway (1969b) for heart glutaminase. On balance it seems likely that glutamine is the major source of the ammonia formed by perfused rat heart.

7.2 Fate of Ammonia

There are three main reactions by which ammonia can enter into combination with organic molecules in higher animals: carbamyl phosphate synthesis, glutamine synthesis and the glutamate dehydrogenase reaction. Of these, only glutamate dehydrogenase has been conclusively demonstrated in rat heart, although glutamine synthetase appears to be present in some other mammalian hearts (Chapter 1, Section 1.2), and possibly also in rat heart (Trush, 1963b).

On perfusion with 300 μmol/l ammonia the concentrations of alanine, glutamine, glutamate and aspartate were not significantly higher than those in hearts perfused without added ammonia, nor could nitrogen balance calculations using these four amino acids account for the ammonia metabolized by the heart. The basis of this discrepancy could not be ascertained. The likely explanation is that a compound not measured in these experiments represents the ultimate fate of the ammonia. Adenine nucleotides are not a possibility since their synthesis requires amination of IMP by aspartate, and there is no measurable IMP in a properly-oxygenated heart (Chapter 5, Section 5.1). In theory increased protein synthesis could account for the lost nitrogen, though it is difficult to see why protein synthesis should increase in response to an increase in free amino acids caused by ammonia perfusion, but not when the increase in amino acids is caused

by perfusion for instance with glutamine, in which case there is no lost nitrogen (Chapter 5, Section 5.2.5.1). Furthermore, incorporation of the nitrogen either into adenine nucleotides or into the peptide backbone of protein requires synthesis of glutamate as the primary event. One would expect this to lead to an increase in tissue glutamate, and a labelling of tissue glutamate from ^{15}N-ammonia, neither of which occurred (Chapter 4, Section 4.3). These observations could only be explained if it is assumed that glutamate, like ammonia (Chapter 4), exists in two intracellular pools, one large and relatively inert, the other small and metabolically active. The concept of compartmentation is further developed below.

Glutamine synthetase has been demonstrated in human, rabbit and guinea-pig heart (Chapter 1, Section 1.2); its absence from rat heart would represent an unusual species-specificity. Perfusion with ammonia caused no increase in glutamine, but this does not exclude the presence of glutamine synthetase since an increase in glutamine was seen on perfusion with glutamate (Chapter 5, Section 5.2.5.2). Possibly net glutamine synthesis can only occur if both the nitrogen and the carbon skeleton are presented at the same time, as is the case with glutamate perfusion:

$$\text{Glutamate} + \text{NAD}^+ + \text{H}_2\text{O} \xrightarrow{\text{GluDH}} \alpha\text{-oxoglutarate} + \text{NADH} + \text{NH}_4^+$$

$$\text{Glutamate} + \text{NH}_4^+ \xrightarrow[\text{Synthetase}]{\text{Glutamine}} \text{Glutamine} + \text{H}_2\text{O}$$

$$2\ \text{Glutamate} + \text{NAD}^+ \longrightarrow \text{Glutamine} + \alpha\text{-oxoglutarate} + \text{NADH}$$

Further information could be gained by perfusing with a medium containing both glutamate and ammonia. It might also be possible to

demonstrate glutamine synthesis conclusively by measuring the labelling of glutamine after perfusion with ^{15}N-ammonia, with or without the addition of glutamate. Until such experiments can be performed, the fate of the assimilated ammonia remains uncertain.

7.3 Transport of Ammonia

Transport of ammonia across cell membranes has generally been considered as one of two processes: the simple diffusion either of uncharged ammonia or of ammonium cation. The present investigation shows that neither of these simple processes satisfactorily explains the observed distribution of ammonia between the tissue and the perfusate. The possibility of a carrier mechanism in addition to one of the simpler diffusion processes was therefore proposed (Chapter 3, Section 3.4.3). A model developed on the basis of work with ^{15}N-ammonia suggested that ammonia entered the tissue by a process displaying saturable kinetics, presumably a carrier (Chapter 4). A carrier mechanism would enable a high tissue ammonia concentration to be maintained even though the blood concentration is considerably lower. A carrier would be a protein molecule, possibly specific to ammonia as may be the case in some lower organisms (Pribil and Kotyk, 1970; Hackette et al., 1970), or possibly also transporting other small cations such as sodium and potassium (Post and Jolly, 1957). In either case the uptake of ammonia against a concentration gradient would require energy. Energy can be provided directly if the transport of cations is coupled to the hydrolysis of ATP, transport and ATPase both being functions of the same protein (Post and Jolly, 1957). Ammonia then enters the cell in exchange for sodium. The physiological role of the

carrier is the maintenance of a high intracellular potassium concentration. Ammonia and potassium thus compete for the same carrier; the effect on such a system of altering the perfusate potassium concentration has already been discussed (Chapter 3, Section 3.4.3). The existence and importance of a carrier mechanism could be further investigated by the addition of the glycoside ouabain to the perfusing fluid. In theory if an ATPase is involved in ammonia transport, its effect should be abolished by this treatment and the resultant ammonia distribution should reflect simple diffusion across the membrane. Variation of the pH or potassium concentration of the perfusing fluid should then give a clearer picture, enabling the mechanism of diffusion to be distinguished either as NH_3 or as NH_4^+ permeability. Alternatively, uncoupling the oxidative phosphorylation of the heart by means of dinitrophenol should abolish all energy-dependent processes including the active transport of ammonia.

Further experiments could be devised to assess the role of organic anions in ammonia permeability. Ammonium salts of certain weak organic acids have been shown to permeate the mitochondrial membrane readily (Chappell, 1968) and there is no reason to suppose that this is any less true of the less specialized plasma membrane. Thus perfusion with media containing acetate, or more physiologically citrate malate or lactate, might accelerate the rate at which ammonia enters the tissue.

Finally, valinomycin is a carrier of potassium ions across cell membranes. It might be used in perfusion as a means of altering the potassium distribution across the membrane, and hence the membrane potential, when any resultant change in ammonia distribution might be observed.

7.4 Compartmentation of Ammonia

Experiments with isotopically labelled ammonia (Chapter 4) suggested that there was a large pool of ammonia which was prevented from exchanging rapidly with the extracellular medium and a smaller pool whose function was to exchange ammonia relatively rapidly with the extracellular fluid, and through which the metabolic formation or disappearance of ammonia occurred. The smaller pool therefore responds to changes in extracellular ammonia, and also deals with any excess tissue ammonia forming for example as a result of hypoxia. The larger pool exchanges very slowly with the smaller. It may be capable of losing ammonia into the external medium, but it cannot take up ammonia from the ECF to any significant extent since this would result in its size being dependent on the external concentration. If it loses ammonia to the ECF it will also require "topping-up" by synthesis from a precursor in the intracellular pool.

The arrangement shown in Fig 7.1 is in accordance with the results of isotopic labelling experiments (Chapter 4). Rate constants derived from these experiments suggest that the precursor (X) of ammonia liberated into perfusate to which none has been added is glutamine, and that most of this ammonia comes from the larger pool (A). Thus on perfusion with glutamine (Chapter 5, Section 5.2.5.1), there is an increased synthesis of ammonia which diffuses out into the perfusate. On perfusion with glutamate there is a significant increase in tissue ammonia, but no increase in perfusate ammonia. It is possible therefore that ammonia is formed from glutamate in the metabolic pool (x), and that glutamine is synthesized from it without any release of ammonia into the perfusate. Compartmentation of tissue ammonia has three implications: firstly, enzymes concerned in ammonia

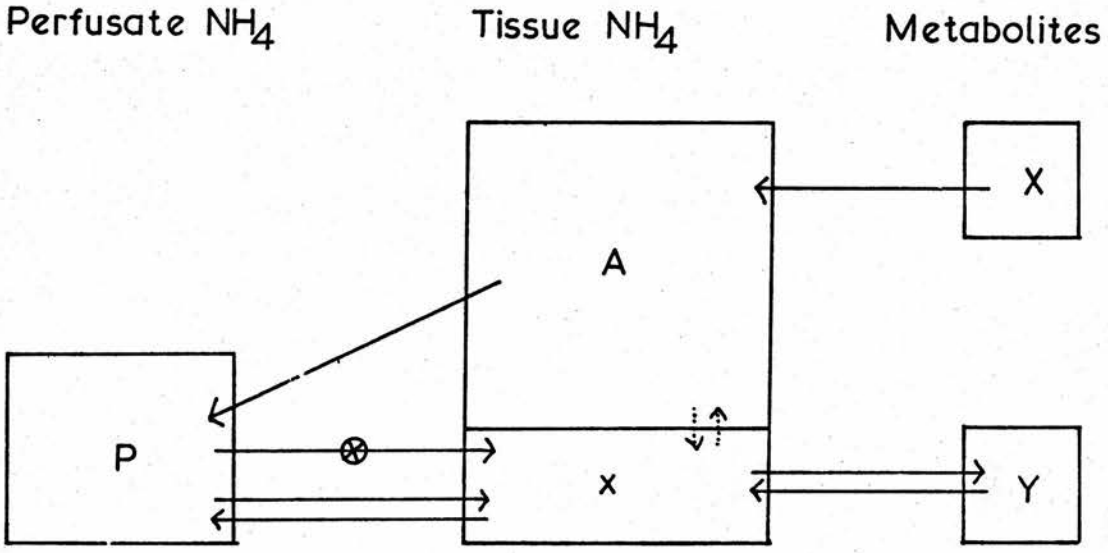


Figure 7.1. Ammonia pools in perfused rat heart.

⟶ = passive diffusion

⟶⊗⟶ = active transport

and amino-group metabolism may also be compartmentally or non-homogeneously distributed; secondly, related metabolites such as glutamate or glutamine may also be compartmental ized; and finally, the compartmental distribution should be related to the known structure of the muscle.

The distribution of GluDH and GOT, the most active enzymes of amino-group metabolism in heart, has been investigated and discussed (Chapter 6). Both enzymes appear to be associated with the I-bands of the myofibrils. GPT was demonstrated in the cytoplasm of the heart, but its activity was too low to permit staining. The possibility of artifacts in the cytoplasmic staining of GluDH and GOT has been discussed; it was suggested alternatively that this staining might represent a small pool of enzyme in the sarcotubular system which in undisrupted tissue is more accessible to external substrates than is the intramitochondrial enzyme, but which on fractional extraction of disrupted tissue would remain with the mitochondrial fraction.

It has been suggested (Chapter 1, Section 1.3) that, if the substrates of the GluDH reaction were homogeneously distributed throughout the cells, the ammonia concentration in the heart would be maintained at a very low level by the action of mitochondrial GluDH, and an ammonia load would cause a toxic depletion of α-oxoglutarate. Compartmentation of tissue ammonia would ensure that at least part of the tissue ammonia did not come into contact with mitochondrial GluDH. If this fraction is located in the cytoplasm, where the NAD^+/NADH ratio is 100 times higher than it is in the mitochondria, it could exist in equilibrium with glutamate, even if it is accessible to a cytoplasmic form of GluDH. It is possible that other substrates

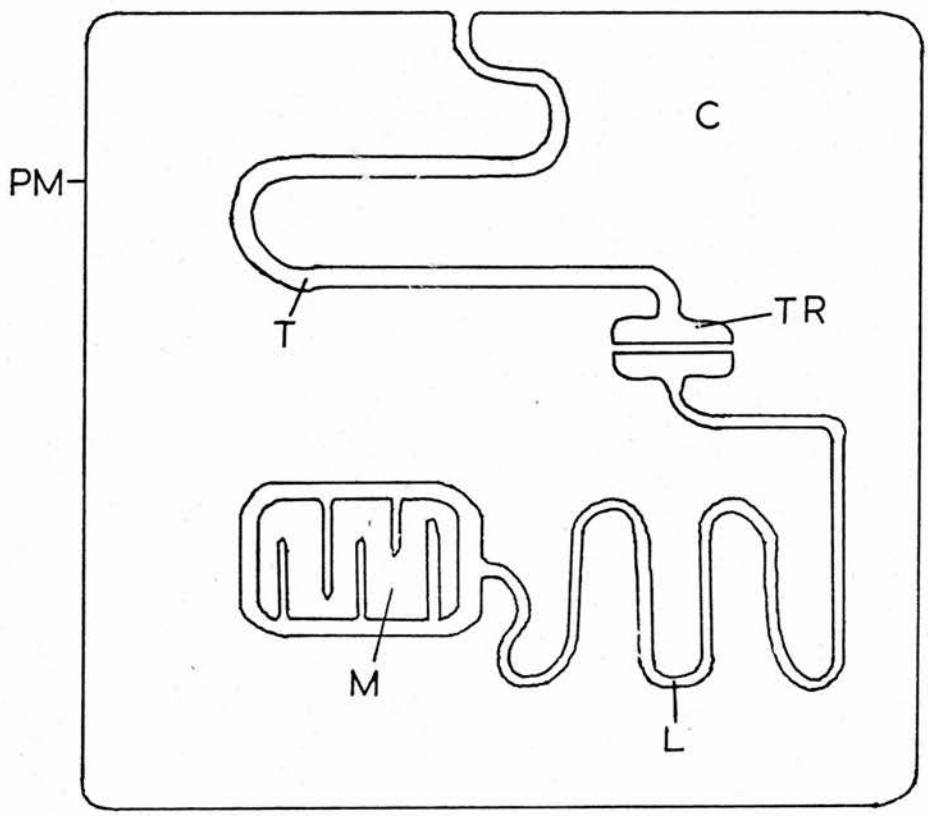


Fig. 7.2 Diagrammatic representation of the T- and L-systems in a heart muscle cell.

C = cytoplasm; L = L-tubule; M = mitochondrion; PM = plasma membrane; T = T-tubule; TR = triad.

concerned in amino-group metabolism, for example amino acids and oxo-acids, occupy a similar compartment. This could be a reason why the labelling of glutamate from ^{15}N-ammonia is less than would be expected if glutamate were formed from ammonia (Chapter 4, Section 4.3).

The location of such a compartment must be related to the structure of the muscle. The simplest form of compartmentation involves a membrane across which ammonia cannot pass. The inner mitochondrial membrane is presumably not such a barrier, since ammonia can cross it easily in the presence of weak organic acids, at least in isolated mitochondria (Chappell, 1968). Muscle, however, has a highly-developed membranous reticulum, consisting of two systems (Chapter 1, Section 1.8). The T-tubules are continuous with the extracellular space (Forssman and Girardier, 1966, 1970; Raynes and Simpson, 1967), and the L-tubules are continuous with the outer mitochondrial membrane (Forssman and Girardier, 1966). The two systems are not themselves continuous, but at defined interfaces or triads the lumen of one is separated from the lumen of the other by a double membrane. The systems may form a route whereby substrates can reach the mitochondria without passing through the cytoplasm (Forssman and Girardier, 1966, 1970). In an active muscle solutes can be moved easily along the lumen of the tubules, the only barrier being diffusion across the membrane at the triadic junctions. Hence a possible basis for the compartmentation of tissue ammonia is shown in Fig 7.2.

The lumen of the L-system is a possible site for the small, actively-metabolized ammonia pool (x). It could exchange readily with the extracellular medium (with which it communicates via the

T-system), and also with the intramitochondrial space. One would expect the metabolism of ammonia catalyzed by mitochondrial GluDH to lead to a production of glutamate when the extracellular ammonia concentration was high, and a labelling of glutamate from ^{15}N-ammonia, neither of which was seen however. It might be that the inner mitochondrial membrane in the intact cell is less permeable to ammonia than the swelling experiments in isolated mitochondria suggest. If this is the case the metabolism of ammonia and related compounds in this compartment could be brought about by small quantities of enzyme in the tubules themselves, probably bound to the membrane, as has already been suggested (Chapter 6, Section 6.3).

The larger, non-exchangeable ammonia pool (A) could be located in the cytoplasm. The rather polar environment of the myofibrillar proteins would tend to hinder the diffusion of ammonia ions through the cytoplasm. Thus the reactivity of pool "x" would be associated with the ease of movement of NH_4^+ in the tubular system, whereas the non-reactivity of pool 'A' would be a consequence of the reduced mobility of NH_4^+ in the cytoplasm. Mowbray and Ottaway (1973) have observed that pyruvate does not mix completely in perfused rat heart. This finding may have a similar structural basis to the compartmentation of ammonia here described.

7.5 Functions of ammonia in heart muscle

In general, ammonia has only been considered as a waste material in higher animals, formed as a by-product of amino acid or nucleotide metabolism. Free ammonia was held to be toxic, so that it must be removed from the tissue as rapidly as possible into the bloodstream, from which it is extracted by the liver and formed into the relatively

harmless urea. Thus high tissue ammonia levels represented a potentially toxic state where the rate of ammonia production exceeded the rate of its elimination. Several properties of ammonia were said to be responsible for its toxicity, chief being its effect on tricarboxylic acid metabolism (Chapter 1, Section 1.3). In the brain it was so toxic (James et al., 1972) that it required removal in situ by glutamine formation. If urea synthesis was insufficient, for example in liver failure, skeletal muscle could take up and metabolize ammonia in order to keep the blood concentration from rising too high (Bessman and Bessman, 1955), thus protecting the brain from damage. Ammonia was supposed to be of no use to the body, except that it formed the basis of a mechanism for hydrogen ion excretion in the kidney (Van Slyke et al., 1943).

However, the high ammonia concentration in the heart is not a consequence of uptake from the blood, as it can be maintained in a rat heart perfused without any added ammonia. Nor did ammonia at the tissue concentrations measured have any demonstrable toxic effect on perfused rat heart, as judged by beating, appearance and oxygen consumption (Chapter 2). Moreover, a tenfold change in extracellular ammonia concentration caused only a twofold change in tissue concentration (Chapter 3). The tissue concentration was kept within limits of 0.3 - 0.7 μmol/g, apparently by three processes: by the synthesis or metabolism of ammonia within the tissue; by active uptake of ammonia from the extracellular medium in which its concentration is much lower; and possibly also by intracellular compartmentation which provided a "store" of ammonia in the tissue. Thus one is led to believe that the ammonia in heart muscle may have some function, and is not merely a waste-product of muscle metabolism.

Ammonia production from adenine nucleotides in skeletal muscle is related to muscle activity (Lowenstein, 1972). The activation by ammonia of enzymes including phosphofructokinase has been discussed (Chapter 1, Section 1.5), and it is feasible that the tissue ammonia concentration plays a role in the regulation of glycolysis in skeletal muscle. However, heart muscle contains relatively more mitochondria than skeletal muscle (Klingenberg, 1964) and its metabolism is more aerobic. It does not have the same requirement to adapt to sudden strenuous bursts of activity by a huge increase in anaerobic glycolysis. Moreover, ammonia in heart muscle is not formed from adenine nucleotides (Chapter 5, Section 5.1), so that ammonia production is not related to the tissue's energy metabolism so closely as it is in skeletal muscle. Hence ammonia in heart muscle may play a part in phosphofructokinase activation, but is of less consequence in regulating the glycolytic flux in this tissue as compared to skeletal muscle.

Nevertheless, there was some evidence that perfusion with 300 μmol/l ammonia caused a slight increase in the oxygen consumption of the perfused heart. It is possible that ammonia aids the mobilization in the cell of weak acid substrates with which it can form poorly-dissociated ammonium salts. Thus it could aid the passage of substrates such as pyruvate and fatty acids across the mitochondrial membrane. Mitochondria have been shown to swell in the presence of ammonium salts of weak acids (Chappell, 1968; Brierley et al., 1970). A similar mechanism could be involved in the diffusion out of the mitochondria of citrate for fatty acid synthesis, and in the diffusion of lactate across the plasma membrane.

There is the further possibility, analogous to events in the kidney, that ammonia is formed by heart glutaminase (Ottaway, 1969a,b) in response to acids such as lactic acid generated by heart metabolism, and that one of its functions is to take up the excess hydrogen ions and prevent the intracellular pH from falling too low. However, no correlation between ammonia and lactate production was found, at least in frog muscle (Embden and Wassermeyer, 1928a). Several methods exist by which the work done by a perfused beating rat heart may be varied (Neely et al., 1967; Krebs, 1970; Opie et al., 1971). In view of the possible relationship of myocardial ammonia to substrate mobilization, it would be of interest to study the effect on ammonia production of varying the work done by the heart.

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[*Reprinted from the Proceedings of the Biochemical Society*, 29–30 *July* 1971.
Biochem. J., 1971, Vol. 125, No. 2, 35P.]

Histochemical Localization of Enzymes in Heart Muscle

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Histochemical localization by formazan deposition, in an agarose gel-film medium, by using phenazine methosulphate to reduce interference from tetrazolium reductases, has been used for extensive studies on the localization of mitochondrial and cytoplasmic enzymes in rat liver (Pette & Brandau, 1966), rabbit skeletal muscle (Sigel & Pette, 1969) and insect flight muscle (Brandau & Pette, 1966). This variant of the tetrazolium localization method has not been used to study the distribution of soluble enzymes in heart muscle.

We now report the results of studies on the distribution of succinate dehydrogenase (EC 1.3.99.1), glutamate dehydrogenase (EC 1.4.1.2), glutamate–oxaloacetate transaminase (EC 2.6.1.1) and lactate dehydrogenase (EC 1.1.1.27) in rat heart muscle. The muscle fibres were caused to relax by soaking the organ in iso-osmotic potassium chloride solution before freezing and sectioning. This pretreatment made it possible to detect the striations of the myofibrils, normally invisible in freshly excised heart tissue. All the enzymes showed a banded distribution, but that for the purely mitochondrial enzymes succinate dehydrogenase and glutamate dehydrogenase was coarser and more irregular, in keeping with the expected localization of these enzymes in mitochondria, which are concentrated, but not exclusively located, opposite the I-bands of the fibrils (Klingenberg, 1964). The picture for glutamate–oxaloacetate transaminase, which is known to occur in both cytoplasm and mitochondria in this tissue, was similar to that for the purely cytoplasmic enzyme lactate dehydrogenase.

It cannot at present be proved conclusively that the 'soluble' enzymes are located within the myofibrils, but the localization pattern appears to be in accord with the hypothesis of a concentrated distribution of many glycolytic and related enzymes around the isotropic zone of skeletal-muscle fibres, which has been put forward by Brandau & Pette (1966) and Sigel & Pette (1969).

This work was carried out in Konstanz as the result of an invitation from D. P., and financial support was provided by the Science Research Council.

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PRINTED IN GREAT BRITAIN BY WILLIAM CLOWES AND SONS LIMITED, LONDON, COLCHESTER AND BECCLES

Histochemical Journal, 7 (1975), 165–178

Localization in cardiac muscle of some enzymes related to glutamate metabolism

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Received 20 August 1974 and in revised form 14 October 1974

Synopsis. A tetrazolium staining medium incorporated in a gel has been used in a histochemical study of enzymes in thin sections of heart muscle. Formazan distribution patterns given by mitochondrial enzymes were inconsistent with the location of these enzymes revealed by the extraction of whole tissue. Similar stain distributions were given by lactate dehydrogenase, glutamate oxaloacetate transaminase and glutamate dehydrogenase. The distribution given by succinate dehydrogenase was not the same as that given by cytochrome oxidase stained by a different technique. Alcohol dehydrogenase added to the tissue assumed a distribution which suggested some adsorption of the enzyme to the tissue, but experiments suggested that this enzyme was not firmly bound to muscle proteins in the manner of some glycolytic enzymes.

Introduction

Ditetrazolium salts have been an important biochemical tool in the study of dehydrogenases in mammalian tissue since the work of Seligman & Rutenberg (Rutenberg *et al.*, 1950; Seligman & Rutenberg, 1951) on succinate dehydrogenase, firstly in tissue homogenates and later in tissue slices. The ease of reduction of the salts and the physical properties of the formazan deposited were improved until the method had become suitable for the accurate localization of insoluble dehydrogenases in tissue slices at the light-microscope level (Farber & Bueding, 1956; Farber & Louviere, 1956; Farber *et al.*, 1956; Nachlas *et al.*, 1957). At that time, however, the technique did not permit the certain localization of freely-diffusible enzymes. Two advances made the study of such enzymes possible. First, the use of soluble redox dyes in the staining medium accelerated hydrogen transfer to the tetrazolium salts and markedly reduced the staining time. This also eliminated the requirement for endogenous tetrazolium reductases, so that the formazan pattern reflected the enzyme studied, irrespective of the distribution of such reductases. A number of these dyes were investigated (Nachlas *et al.*, 1957); phenazine metho-

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sulphate is now almost universally used. Secondly, incorporation of the staining system into a gel-film which was applied to the tissue slice reduced the leakage of soluble enzymes out of the tissue. Gelatine was first used (Fahimi & Amarasingham, 1964), but is now generally replaced by agarose. Using this latter technique a series of studies has been made on soluble enzymes in rat liver (Pette & Brandau, 1966), insect striated muscle (Pette & Brandau, 1962; Brandau & Pette, 1966) and rabbit skeletal muscle (Sigel & Pette, 1969). The gel-film method has not been used to study the distribution of soluble enzymes in heart muscle, although structure-bound enzymes such as succinate dehydrogenase and ATPase have been studied in some detail (Niles *et al.*, 1964; Morales & Fine, 1965; Chayen *et al.*, 1966; Niles *et al.*, 1966). The present work represents a study of the distribution of succinate dehydrogenase, glutamate dehydrogenase, lactate dehydrogenase and glutamate-oxaloacetate transaminase in rat heart muscle. For glutamate-oxaloacetate transaminase a method has been evolved in which reaction is coupled to the production of formazan in the reaction catalysed by glutamate dehydrogenase. A preliminary report of this work has already appeared (Jarvie *et al.*,1971).

Materials and methods

The following biochemicals were obtained from Boehringer und Soehne GmbH, Mannheim: enzymes and nucleotides, cytochrome *c*, sodium L-lactate, and sodium succinate, L-aspartic, L-glutamic and α-oxoglutaric acids and triethanolamine hydrochloride. Phenazine methosulphate, nitro-BT, diaminobenzidine tetrahydrochloride, and agarose were obtained from Sigma Corporation. Other reagents and inorganic chemicals were obtained either from BDH biochemicals, Poole, Dorset, or from E. Merck, Darmstadt, Germany. Lyophilized alcohol dehydrogenase was obtained from BDH biochemicals.

Preparation of tissue

The procedure was based on the method of Pette (Pette & Brandau, 1966; Sigel & Pette, 1969). Rats (laboratory strain) were fed on a normal diet and killed by cervical fracture and exsanguination. The heart was removed as quickly as possible and transferred to ice-cold isotonic potassium chloride solution; when contraction ceased, the heart remained in a relaxed state. The organ was cut into strips of about 2 mm cross-section and returned to the KCl for a further 1 min. The strips were then rapidly blotted and frozen in isopentane precooled in liquid nitrogen. No contraction was apparent on freezing. Sections of 10 μm thickness were cut at −20°C with a microtome mounted in a cryostat (W. Dittes, Heidelberg, Germany), as described by the above authors. Suitable sections for staining were selected by examination in the phase-contrast microscope. In one experiment, the hearts were perfused (10 min) with an ice-cold Ringer in which the NaCl (138 mM) had been replaced by an equivalent concentration of KCl. The resultant tissue sections presented an identical microscopic appearance to those prepared by soaking the whole organ in the solution.

Preparation of gel-films

Gel-films were prepared and stored as described by Pette & Brandau (1966). Gel media (1.5% agarose) were used for all enzymes, including succinate dehydrogenase. The com-

position of staining media was as follows (values refer to the final concentrations of reagents in the gel-film). Test media for lactate, glutamate and succinate dehydrogenases are as follows:

Lactate dehydrogenase (1.1.1.27). Triethanolamine/triethanolamine hydrochloride buffer (TRE) = 50 mM, pH = 7.6; EDTA = 1 mM; nitro-BT = 1.5 mM; NAD^+ = 2.3 mM; L-lactate = 24 mM and phenazine methosulphate (PMS) = 0.3 mM.

Glutamate dehydrogenase (1.4.1.2). TRE = 50 mM; pH = 7.6; EDTA = 5 mM; nitro-BT = 1.5 mM; $NADH^+$ = 2.3 mM; L-glutamate = 50 mM; ADP = 2 mM; PMS = 0.15 mM.

Succinate dehydrogenase (1.3.99.1). Sodium phosphate = 100 mM; pH = 7.6; EDTA = 5 mM; nitro-BT = 1.5 mM; PMS = 0.15 mM; succinate = 80 mM; KCN = 1 mM.

Glutamate-oxaloacetate transaminase (2.6.1.1). The staining medium involved the coupling of transamination to glutamate reduction by the reactions:

Test reaction: α-oxoglutarate + aspartate $\rightarrow$ glutamate + oxaloacetate.

Auxiliary reaction: glutamate + $NADP^+$ + $H_2O \rightarrow NH_4^+$ + α-oxoglutarate + NADPH.

Glutamate dehydrogenase is incorporated in the gel as the auxiliary enzyme, and formazan deposition occurs at the site of the transaminase in the tissue.

Appropriate concentrations of reagents were found by tests in solution, the relative rates of tetrazolium reduction being followed spectrophotometrically. α-Oxoglutarate is a substrate of the test-reaction and a product of the auxiliary reaction. Its optimal concentration was found to be 1.5 mM. ADP was added as an activator of glutamate dehydrogenase: it had no effect on the transaminase reaction *in vitro*. $NADP^+$ was used as the coenzyme for the auxiliary reaction, since NADH is reoxidized by the oxaloacetate formed in the test reaction (rat heart contains a high concentration of malate dehydrogenase). Glutamate dehydrogenase was dialysed before addition to the gel, to remove ammonium sulphate. Staining was complete in 30 min. Control gels lacking aspartate did not stain.

Medium: TRE = 50 mM; pH = 7.6; EDTA = 5 mM; nitro-BT = 1.5 mM; $NADP^+$ = 5 mM; α-oxoglutarate = 1.5 mM; L-aspartate = 50 mM; glutamate dehydrogenase = 100 i.u./ml; ADP = 2 mM; PMS = 0.2 mM.

Gel slices were applied to tissue sections as described by Pette & Brandau (1966), and incubated at 37°C in the dark until sufficient colour had developed, as judged by rapid inspection under the microscope. On average, this required 10 min for succinate and lactate dehydrogenases, and 30 min for glutamate oxaloacetate transaminase and glutamate dehydrogenase. After removal of the gel slice, the sections were fixed in 4% formaldehyde solution, dried and mounted in glycerol-gelatine.

Localization of cytochrome oxidase

Cytochrome oxidase was localized using the technique of Seligman (1968). Tissue sections were prepared on microscope slides by the technique described above, and 100–200 μl staining medium was pipetted on to each. After 40 min incubation at 25°C, the section was carefully blotted, fixed in 5% acetic acid, dried and mounted in glycerol-gelatine.

The medium was made up as follows: sodium phosphate = 0.05 M, pH 7.4; diamino-

benzidine tetrahydrochloride (Sigma) = 0.5 mg/ml; catalase = 2 μg/ml; cytochrome *c* = 1 mg/ml; sucrose = 75 mg/ml (0.22 M). Control gels contained in addition 10 mM potassium cyanide to inhibit cytochrome oxidase. Colour developments in control sections were not completely prevented unless the tissue sections were preincubated for 5 min in a medium containing buffer, 75 mg/ml sucrose and 10 mM KCN (Nir & Seligman, 1971).

Adsorption test with exogenous enzyme

In experiments designed to study the possible adsorption of exogenous alcohol dehydrogenase to tissue, slices were cut and transferred to microscopic slides in the normal manner. Before application of the staining gel, a 5 μl drop of solution containing yeast alcohol dehydrogenase (or a similar drop of a control solution) was placed on the tissue slice. After 3 min incubation at room temperature, excess liquid was soaked away with tissue and a staining gel for visualization of alcohol dehydrogenase was applied to the slice. Staining was sufficient in 10 min at room temperature (or in 5 min at 37°C). Since the technique loosens the tissue from the slide, it was no longer possible to remove the staining gel and to fix and mount the section. Sections were, therefore, photographed immediately on development of the stain, with the gel slice in position. Alcohol dehydrogenase solutions contained 10 mg protein per ml and were applied at two different ionic strengths: (1) in 0.8 M ammonium sulphate solution and (2) after overnight dialysis against two changes of 2 mM phosphate, pH 7.0. In control experiments, drops of distilled water or drops of 0.8 M ammonium sulphate were applied to the tissue slices.

A further experiment tested for the adsorption of non-enzymic components of the staining system to the tissue. In this test, a 5 μl drop of NADH solution (15 mM) was applied to the tissue slice for 3 min and then soaked away with tissue paper before application of the appropriate staining gel.

The staining gels required were as follows:

Alcohol dehydrogenase (1.1.1.1). TRE = 50 mM, pH = 7.6; nitro-BT = 1.5 mM; NAD = 3.0 mM; Ethanol = 44 mM; PMS = 0.15 mM.

NADH stain. TRE = 50 mM; pH = 7.6; EDTA = 5 mM; nitro-BT = 1.5 mM; PMS = 0.15 mM.

Enzyme localization in disrupted tissue

In this investigation, the 'fractional extraction' technique of Pette (1968) was used. Freshly-excised heart was minced in a specially constructed micro-meat-mincer. The tissue was gently extracted in two changes of sucrose medium (0.3 M sucrose, 10 mM TRE, 2 mM EDTA, pH 7.4), yielding supernatants A and B, followed by one extraction in 0.1 M phosphate buffer, pH 7.2, giving supernatant C. Finally, the solid material was homogenized in the same buffer (Polytron PT 10 Homogenizer, Kinematic, Luzern, Switzerland), yielding supernatant D and the pellet S. Enzyme activities were measured in standard spectrophotometric assays (Bücher *et al.*, 1964).

In vitro *extraction pattern of exogenous alcohol dehydrogenase*

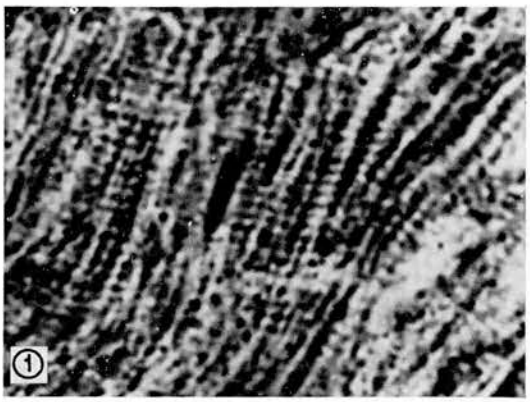
In testing for adsorption of alcohol dehydrogenase in soluble components of a rat heart homogenate, a freshly excised heart was washed for 10 min by coronary perfusion with ice-cold Ringer (Krebs & Henseleit, 1932). Tissue (0.5 g) was homogenized (Ultraturrax

homogenizer, Janke & Kunkel) in a medium of low ionic strength similar to that used in the fractional extraction of endogenous enzymes (0.3 M sucrose, 5 mM TRE, 0.5 mM $MgSO_4$, pH 7.4). Yeast alcohol dehydrogenase was added (0.1 mg of lyophilized powder, containing 8.40 enzyme units as assayed), and the total volumes were made up to 10 ml with sucrose medium. The mixture was stored for 20 min at 0°C to allow binding of ADH to take place before centrifuging at 36000 ***g*** for 25 min. The sediment was washed with a second 10 ml portion of sucrose medium, then with 0.1 M phosphate buffer (high ionic strength), pH 7.2. In each case 15 min stirring preceded the centrifugation. Alcohol dehydrogenase activity in all fractions was assayed by the method of Racker (1950), with the addition of semicarbazide hydrochloride (7.5 mM) and glutathione (0.98 mM).

Results

The method of tissue preparation produced relaxed fibres, as evinced by the striation seen on staining (Figs. 1–3), which were also seen when viewed by polarized light. Regions of fibrils without striations may have been due to incompleteness of relaxation though all fibres which showed striations seemed to be relaxed to the same extent as judged by the distance between striations (Table 1).

Staining patterns for the endogenous enzymes are shown in Figs. 1–6. Formazan was not deposited in control sections incubated in the absence of substrate. All the enzymes



Figures 1–4. Distribution of enzymes in sections of cardiac muscle stained by the tetrazolium technique as described in the text. Staining times are given in parentheses. × 760

Figure 1. Lactate dehydrogenase (10 min).

Figure 2. Glutamate dehydrogenase (30 min).

Figure 3. Glutamate oxaloacetate transaminase (30 min).

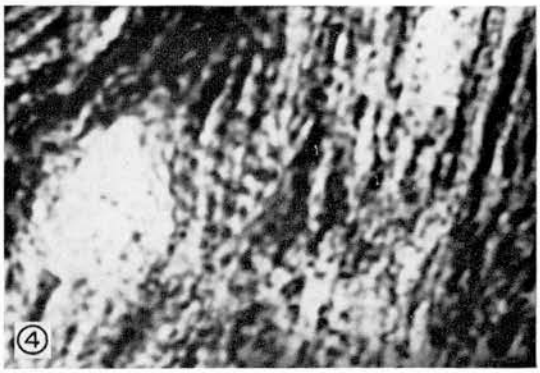
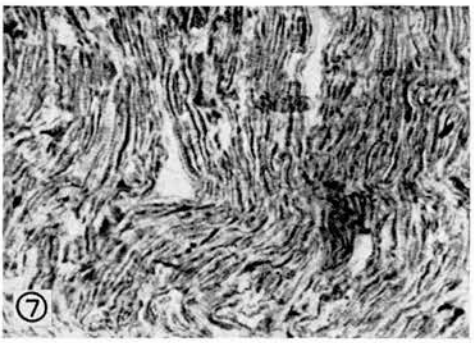


Figure 4. Succinate dehydrogenase (10 min). Note the similarity in the patterns given by the first three enzymes, and the anomalous pattern given by succinate dehydrogenase.

Figures 5 & 6. Comparison of the stain distribution given by two structure-bound enzymes. × 310

Figure 5. Succinate dehydrogenase, stained by the tetrazolium technique (10 min)

Figure 6. Cytochrome oxidase, stained by the diaminobenzidine technique (40 min). Cytochrome oxidase gives a pattern consistent with localization of the enzyme in the interfibrillar mitochondria; succinate dehydrogenase does not yield the expected pattern.



Figures 7 & 8. Controls to test for possible adsorption of enzyme and components of the staining system to elements of the tissue. × 270

Figure 7. The pattern of formazan obtained on staining a tissue, previously suffused with alcohol dehydrogenase, with a staining gel for localization of this enzyme (5 min). Some striations are visible, suggestive of fibrillar adsorption, but the predominant dark bands suggest that much of the enzyme assumes a location in the interfibrillar spaces.

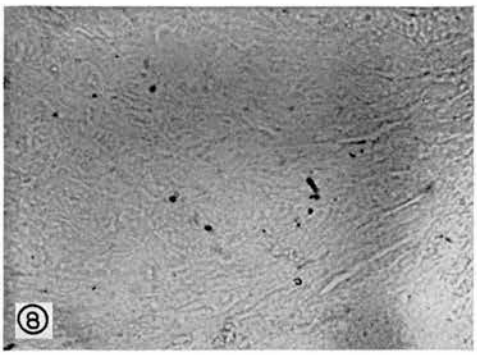


Figure 8. The pattern obtained on staining a tissue previously suffused with NADH (10 min). There are no formazan granules or striations in the tissue; thus adsorption of NADH or other components of the staining system can be excluded.

except succinate dehydrogenase and cytochrome oxidase gave a similar pattern, characterized by fibrillar staining with distinct transverse striations. Succinate dehydrogenase (Figs. 4, 5) gave a markedly different distribution, with significantly wider striations (Table 1). The pattern of this enzyme was similar to that observed by Chayen and his group (Chayen *et al.*, 1966; Niles *et al.*, 1966), though the granular stain distribution observed by these authors in the presence of PMS was not found. Cytochrome oxidase gave a pattern in which very few striations were discernible (Fig. 6), consistent with location of the enzyme in the interfibrillar mitochondria.

Fig. 7 shows the formazan deposition in a tissue slice treated with exogenous alcohol dehydrogenase in a medium of low ionic strength. A similar pattern was obtained when the enzyme was applied in a medium of high ionic strength. The formazan distribution is superficially similar to that given by endogenous lactate dehydrogenase, glutamate dehydrogenase and glutamate oxaloacetate transaminase; some striations are visible, suggestive of limited fibrillar adsorption. However, the predominant dark bands suggest that most of the enzyme assumes a location in the interfibrillar spaces. Treatment of tissue slices with NADH (Fig. 8) did not give rise to any specific tissue staining: formazan was deposited homogeneously in the staining gel and in the section. Control experiments in which water or 0.8 M ammonium sulphate was used in place of alcohol dehydrogenase, produced no stain.

The dimensions of the patterns were measured in half-plate prints and compared with measurements made in photographs published by other authors (Table 1). The ratio r in Table 1 is the ratio of the average striation width to the average distance between

Table 1. Dimensions of banding patterns in heart myofibrils stained by the tetrazolium technique.
(*a*)

Enzyme	*Figure*	*l* (μm)	*r*
Lactate dehydrogenase	1	2.3	0.81
Glutamate dehydrogenase	2	2.3	0.86
Glutamate oxaloacetate transaminase	3	2.3	0.78
Alcohol dehydrogenase (added)	7	2.3	0.91
Succinate dehydrogenase	4	4.3	1.32
Succinate dehydrogenase	5	4.1	1.32

Sarcomere length = 2.5 μm. (Bloom & Fawcett, 1968)

(b) *Literature values (for succinate dehydrogenase)*

r	*Reference*
0.86	Chayen *et al.*, 1966
1.39	Burstone, 1962
1.40	Nachlas *et al.*, 1957
1.43	Nachlas *et al.*, 1957
1.54	Novikoff, 1959

l is the distance between successive dark striations, *r* is the ratio of *l* divided by the distance between adjacent fibrils. *l* was obtained by measuring 10 consecutive striations along a fibril and dividing by 10; this procedure was repeated at least 5 times for each photograph and the mean was calculated. The mean distance between fibrils was obtained in an analogous manner. Literature values were obtained by making similar measurements on published photographs. For these only the ratio is reliable, since in many cases the actual magnification of the published picture is uncertain.

adjacent fibrils, and enables comparison with photographs in the literature whose exact magnification is uncertain. The length *l* is the actual width of the striations in microns, which corresponds to the sarcomere length (given as 2.5 μm by Bloom & Fawcett, 1968) for all the enzymes except succinate dehydrogenase.

Fractional extraction of heart muscle according to the method of Pette (1968) gave the results shown in Table 2. Lactate dehydrogenase is wholly cytoplasmic, glutamate dehydrogenase wholly intramitochondrial, whereas glutamate oxaloacetate transaminase is distributed between both compartments. This is comparable to the findings of Pette (1968) in insect flight muscle. Succinate dehydrogenase is bound to structural protein and sediments with the final pellet.

The results of extraction of alcohol dehydrogenase, by a method analogous to fractional extraction, from a homogenate to which it has been added are shown in Table 3. The whole of the enzyme activity added was extractable by a medium of low ionic strength, and thus no binding of the enzyme to insoluble components could be demonstrated.

Table 2. Fractional extraction of enzymes in rat heart. See Pette (1968) and Table 3 for explanation of fractions.

Enzyme	*Total activity (μmol/min/g fresh weight at 25°C)*	*Cumulative percentage of total activity extracted*				
		S_1	S_2	S_3	S_4	P
Lactate dehydrogenase	478.4	96.7	98.3	98.7	99.9	100.0
Glutamate pyruvate transaminase	0.78	68.7	100.0	100.0	100.0	100.0
Malate dehydrogenase	1309.5	33.9	36.9	43.0	95.7	100.0
Glutamate oxaloacetate transaminase	196.8	25.0	28.2	31.4	93.8	100.0
Citrate synthetase	56.6	4.9	5.2	9.7	87.5	100.0
Glutamate dehydrogenase	12.9	5.0	7.5	12.5	88.2	100.0
Succinate dehydrogenase	26.7	0.0	0.0	0.1	1.1	100.0
Protein	118.5 mg/g	23.4	26.5	30.0	42.5	100.0

Table 3. Adsorption of yeast alcohol dehydrogenase to a rat heart homogenate. 0.5 g rat heart was homogenized in 10 ml sucrose medium (see text). 8.40 units of alcohol dehydrogenase (1 unit = 1 μmol of ethanol per min at 25°C) were added and the mixture was stirred gently at 0°C for 20 min. Centrifugation yielded supernatant S_1 snd a pellet, which was resuspended in sucrose medium and stirred for 15 min. The resultant supernatant was S_2. A subsequent 15 min wash of the pellet with 0.1 M phosphate buffer, pH 7.2, yielded supernatant S_3 and the pellet P.

	Alcohol dehydrogenase activity extracted (μmol/min at 25°C)	*% of total activity added*
Fractions S_1	7.27	86.5
S_2	1.19	14.2
S_3	0.00	0.0
P	0.00	0.0
Total activity recovered	8.46	—
Recovery (%)	—	100.7

Discussion

The purpose of this investigation was to localize, if possible, in mammalian heart muscle, glutamate oxaloacetate transaminase and glutamate pyruvate transaminase. The other enzymes were stained for as controls to check the validity of the results for glutamate oxaloacetate and glutamate pyruvate transaminases. de Duve *et al.* (1962) have already recorded that lactate dehydrogenase is always a purely cytoplasmic enzyme, whereas succinate dehydrogenase and glutamate dehydrogenase are mitochondrial enzymes, and both have been used as markers for mitochondria in biochemical separation procedures. Like malate dehydrogenase, glutamate oxaloacetate transaminase has been detected both in mitochondria and in cytoplasm of various animal tissues, and this

dual localization is an essential part of a mechanism currently proposed for the transfer of reducing equivalents into mitochondria (see, for example, Safer & Williamson, 1973).

Glutamate pyruvate transaminase was found to be solely localized in the cytoplasm by the fractional extraction technique (Table 2), thus confirming Pette's (1962) results for beef heart. Unfortunately, its concentration in heart muscle is too low to give staining by the method successfully developed for glutamate oxaloacetate transaminase.

The distribution of dehydrogenases, and of those glycolytic enzymes which may be coupled to them, have been extensively studied by the tetrazolium gel-staining technique (Pette & Brandau, 1962, 1966; Brandau & Pette, 1966; Sigel & Pette, 1969). In striated muscle from several sources the distribution of many glycolytic enzymes has been found to be banded, with heavy staining at the level of the I-bands of the muscle fibrils. Lactate dehydrogenase is the type enzyme showing this distribution pattern. In some instances, this banding can be correlated with demonstrable physical binding of the enzymes to fibrillar components, notably actin (Arnold & Pette, 1968, 1970; Arnold *et al.*, 1969, 1971). These studies have not previously been extended to cardiac muscle, but the banded distribution of lactate dehydrogenase which we observed in rat heart muscle (Fig. 1) agrees in every particular with that found by Pette's group for other muscle types.

In some skeletal muscles, the interfibrillar mitochondria tend to be arranged in pairs at the level of the I-bands, but in heart muscle they form continuous columns completely filling the interfibrillar space (Klingenberg, 1964). Thus it would be expected that the stain produced in heart muscle sections by a completely intramitochondrial enzyme would be distributed as lines of dots, perhaps even largely fused together, running parallel to the direction of the fibrils, which can be distinguished by phase-contrast microscopy. With cytochrome oxidase, stained by the diaminobenzidine technique, this was the case (Fig. 6), but the two other mitochondrial marker enzymes succinate and glutamate dehydrogenases, did not give the expected pattern (Figs. 5, 2). Succinate dehydrogenase appeared to be located in granules somewhat clumped together, and separated by blank spaces. These quasi-uniform regions were only in register over short distances and the repeat distance was almost twice the distance between the I-bands in relaxed muscle (see Table 1). The significance of this observation cannot at present be assessed, but we are confident that it is not due to overstaining (see below). Similar findings have been reported by Chayen *et al.* (1966) and Niles *et al.* (1966).

The staining pattern found for glutamate dehydrogenase (Fig. 2) differed considerably from this. It was surprisingly similar to the banded distribution characteristic of lactate dehydrogenase, with scattered granules which might be identified with mitochondria superimposed on it. The bands were in register over several fibril widths for long distances, and the spacing of the bands corresponds to the relaxed sarcomere length (Table 1). The staining pattern for glutamate oxaloacetate transaminase was not dissimilar, but with fewer scattered granules. These results were so surprising that a test was made by flooding the section, before applying the gel, with a solution of alcohol dehydrogenase, a soluble enzyme not found in heart muscle (Morales & Fine, 1965). A weak, but distinct, banded pattern was also found for this enzyme (Fig. 7), with a repeat distance of 2.3 μm.

The four enzymes (succinate, alcohol and glutamate dehydrogenases, and glutamate oxaloacetate transaminase) which gave unexpected staining patterns, were all localized by

the tetrazolium technique, and this inevitably casts some suspicion on the method. The possibility that the results are an artefact of formazan deposition, as opposed to production, can almost certainly be set aside. The deposition of non-specific granules seems from inspection of the photomicrographs, most likely to have occurred with staining for succinate dehydrogenase (Fig. 4). However, visual control of staining enabled us to check that the pattern did not alter significantly over the total staining time. It is difficult to explain the regularity with which the granules appear at least over short distances in terms of non-specific deposition, or the fact that very similar ratios of repeat distance to interfibrillar space to those found by us have been reported by several other workers (Table 1). Table 1 also shows that the repeat distance for succinate dehydrogenase does not correspond with the sarcomere length, as it did for the other enzymes which we investigated. We are unable to explain this.

We also found that when a section was flooded with NADH, the formazan was subsequently deposited uniformly (Fig. 8), in distinction to the pattern found when it was flooded with alcohol dehydrogenase and ethanol (Fig. 7). Moreover, Pette and coworkers have demonstrated very strict mitochondrial localization of succinate and glutamate dehydrogenases in locust muscle (although admittedly not in columnar mitochondria), and, on the other hand, have shown that malate dehydrogenase, 'malic enzyme' and NADP-linked isocitrate dehydrogenase, which are all thought to be both cytoplasmic and mitochondrial, show an interfibrillar distribution in locust flight muscle which is quite different from the striated appearance of Figs. 2 & 3. Much of the study reported here was carried out in Pette's laboratory, and it is unlikely that it departed much from his own workers in points of technique. Thus we may accept that striations at the I-band level are not an inevitable consequence of the tetrazolium gel slice procedure.

It is also possible that the treatment of the tissue in some way damaged the mitochondria, and that the enzymes released from the matrix as a result of the damage, instead of diffusing throughout the cytoplasm, attached themselves to some insoluble component of the I-band structure. The experience with alcohol dehydrogenase shows that this may be possible, although it is evident that the binding of this enzyme to a fibrillar structure is different from the binding of the glycolytic enzymes studied by Arnold *et al.* (1971), since the latter were not bound at high ionic strengths, whereas alcohol dehydrogenase binding to the section was unaffected by 0.8 M ammonium sulphate. Moreover alcohol dehydrogenase, unlike glycolytic enzymes, does not appear to bind to any component of disrupted muscle.

Although it is possible that the banded patterns found with glutamate dehydrogenase and glutamate oxaloacetate transaminase were artefacts of preparation of the heart sections, the proposition is not easy to believe. It implies that almost all the mitochondria were disrupted, that almost all of them lost the major portion of their contents, and that the glutamate oxaloacetate transaminase and glutamate dehydrogenase (and perhaps other matrix enzymes) became overwhelmingly attached to an I-band component. In the present state of our knowledge, these premises cannot be ruled out, but the first two at least seem rather unlikely. There is no reason to suppose that soaking the heart in isotonic KCl damages the mitochondria, since this medium is used by some biochemists to study their permeability, and heart mitochondria are particularly resistant to freezing and thawing. Moreover, the fact that sections stained with diaminobenzidine had to be pre-incubated with KCN suggests that the mitochondrial membrane was well preserved.

The possibility must, therefore, be at least considered that the histochemical studies are correct, and that the purely biochemical data are, to some extent, misleading. Two consequences may be induced.

(*a*) Impressive evidence has been accumulated that many enzymes of the Embden-Meyerhof pathway, and also enzymes of glycogen metabolism, are concentrated in the I-band region of striated muscle (Arnold & Pette, 1969). It seems not unreasonable that a fraction of the glutamate oxaloacetate transaminase and glutamate dehydrogenase of heart muscle could also be located here, since glutamate and aspartate have been implicated in transfer of reducing equivalents between the glycolytic system and the electron transport chain. If this non-mitochondrial fraction of the two enzymes is located in the T-tubules or in the sarcoplasmic reticulum, rather than being bound to F-actin, it would be possible for it to be spun down with the mitochondria in the differential extraction procedure, and assumed to have arisen from the latter source.

(*b*) The staining patterns shown in Figs. 2 & 3 imply that the mitochondrial glutamate dehydrogenase and glutamate oxaloacetate transaminase are not equally distributed among the mitochondria filling the interfibrillar spaces in cardiac muscle (unless some mitochondria are impermeable to PMS or nitro-BT). Unequal distribution is a very unorthodox proposal; since mitochondria are thought to reproduce themselves by budding, they are all likely to have identical enzymic complements. There is, however, a certain logic about the supposition that the paired mitochondria opposite the I-bands in skeletal muscles (Klingenberg, 1964), whose primary function in this tissue may well be the oxidation of pyruvate and NADH arising from glycolysis, retain this position and function in cardiac muscle, while the regions opposite the A-bands have become filled with mitochondria specialized for fat oxidation and longer-term energy provision for the heart muscle as a whole. As these mitochondria need not respond to the large flux of pyruvate which follows the increased glucose utilization of exercise, their content of pyruvate oxidase, and perhaps of enzymes of amino acid metabolism, might be lower than normal. They might indeed have a reduced content of enzymes of the tricarboxylic acid cycle, since 40% of the energy released in the oxidation of fatty acids appears in the β-oxidation steps, and not in the oxidation of acetyl residues. These speculations might explain the banded pattern seen in Figs. 2 & 3. Mowbray & Ottaway (1973, and in press) have shown, from a study of isotopic labelling patterns in rat hearts perfused with radioactive lactate, that pyruvate does not mix completely within the heart, and that there appear to be some mitochondria oxidizing pyruvate which does not derive from lactate.

These ideas can only be tentatively put forward at present, because the experimental evidence is far from complete. Until both a dehydrogenase known to be restricted to mitochondria, and a dehydrogenase at least partially cytoplasmic (perhaps malate dehydrogenase) can be shown to give a columnar (interfibrillar) staining pattern in heart muscle sections with the tetrazolium gel slice technique, the possibility must remain that the results reported here are technical artefacts. If this proves to be the case, all the evidence for the location of glycolytic enzymes in the I-bands of striated muscle will have to be closely re-examined.

Acknowledgements

Part of this work was carried out in the University of Konstanz. D. R. Jarvie gratefully

acknowledges personal tuition by Professor D. Pette and Dr J. Nolte in many of the techniques used. Financial support was provided by the Science Research Council.

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